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Estrogens are required for mammary epithelial proliferation and are prerequisite for breast cancer formation. Accordingly, strategies to down-regulate estrogen activity have been proven effective to treat and prevent breast cancer. A major pathway to deactivate estrogens is through estrogen sulfation mediated by the phase II sulfotransferases (SULT). SULT is expressed in normal mammary epithelial cells but its expression and activities are significantly lower in many breast cancer cells, suggesting that down-regulation of SULT may led to unchecked estrogen stimulation and cancerous transformation. The expression of sulfotransferase is regulated by orphan nuclear receptor PXR. We hypothesize that the lower SULT activity in breast cancer cells is due to lower expression and activation of the PXR receptor. Activation of PXR by chemical or genetic means will induce the expression of SULT, which in turn facilitates estrogen deactivation and inhibits estrogen-dependent breast cancer cell growth. The traditional antiestrogenic agents such as tamoxifen, though effective, are known to have untoward clinical side effects. It is anticipated that development of PXR activating and ER neutral agents may represent a novel strategy to functionally deprive estrogen activity and to treat and prevent breast cancers.

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INTRODUCTION

Breast cancer and estrogens. Breast cancer is a leading cause of death in American women. Estrogens are required for proliferation of mammary epithelial cells and are prerequisite for breast cancer formation. Accordingly, strategies to down-regulate estrogen activity, such as inhibition of estrogen receptor (ER) binding and estrogen deprivation, have been proven effective to treat and prevent breast cancers.

Estrogen sulfotransferase (EST) in estrogen deactivation and breast cancer. A major metabolic pathway to deactivate estrogens is through the EST-mediated sulfation since the sulfonated estrogens cannot bind and activate the ER (Falany et al., 2002). The human EST belongs to a family of cytosolic sulfotransferases that are critical for the detoxification and clearance of many steroid hormones including estrogens. EST is highly expressed in human mammary epithelial (HME) cells but its expression is significantly lower in many breast cancer cells, including the ER positive and estrogen responsive MCF-7 cells (Falany et al., 2002). The differential expression of EST in normal HME and breast cancer cells suggested that down-regulation of EST may led to unchecked estrogen stimulation and cancerous transformation of the breast epithelium.

PXR, an orphan nuclear receptor that regulates sulfotransferases. PXR functions as the primary xenobiotic receptor to regulate the expression of genes that encode drug metabolizing enzymes including sulfotransferases (Xie et al., 2000a; 2000b; Sonoda et al., 2002). The regulation is achieved by binding of the receptor to PXR response elements found within the promoters of target genes. PXR is expressed in the normal and neoplastic breast tissues (Dotzlaw et al., 1999). Moreover, the expression of PXR varies among breast tumors. The levels of PXR mRNA in ER positive tumors were significantly lower that those observed in the ER negative tumors (Dotzlaw et al., 1999), suggesting a role of PXR in breast cancer pathogenesis. However, whether or not the low PXR expression accounts for the low EST activities in breast tumors is unclear.

BODY

RATIONALE OF THIS STUDY: Members of the sulfotransferase family have been shown transcriptionaly regulated by PXR. We speculate that the lower EST activity in breast cancer cells is due to lower expression and/or insufficient activation of the PXR receptor. Accordingly, we expect that activation of PXR by chemical or genetic means will induce the expression of EST through the PXR response elements present in the EST gene promoter. The increased expression of EST in turn facilitates estrogen deactivation and inhibits estrogen-dependent breast cancer cell growth. The traditional antiestrogenic agents such as tamoxifen, though effective, are known to have untoward clinical side effects, such as risk for endometrial cancer and deep vein thrombosis. It is anticipated that development of PXR activating and ER neutral agents may represent a novel strategy to functionally deprive estrogen activity and to treat and prevent breast cancers.

OBJECTIVES AND METHODS: The overall objective is to establish the human EST as a transcriptional target of PXR, and to determine the effects of PXR activation and EST induction on estrogen-dependent breast cancer cell growth. We propose the following specific aims:

1. To determine whether EST is a transcriptional target of PXR

The human EST gene promoter will be cloned by PCR and its activation in MCF-7 cells by PXR will be examined by co-transfection of promoter reporter genes and PXR, followed by exposure to PXR agonists. The PXR- responsive promoter sequences will be

inspected for putative PXR response elements. DNA-receptor binding (gel shift) and reporter gene analysis will be performed to determine whether these elements are both necessary and sufficient to mediate the transactivation by PXR (Xie et al., 2000a; 2000b; Sonoda et al., 2002). We predict that EST can be transactivated by PXR via the binding of this receptor to its response elements present in the EST gene promoter.

2. To determine the effects of PXR activation on EST expression and estrogen-dependent breast cancer cell growth

MCF-7 cells that stably overexpress the wild type hPXR or its activated variant VP-hPXR (Xie et al., 2000a; 2000b; Sonoda et al., 2002) will be created. The hPXR expressing cells will be treated with known PXR agonists to determine whether chemical (ligand) or genetic activation of PXR is sufficient to induce EST expression in MCF-7 cells. EST activity in the cytosolic fractions will be measured using estrogen as the substrate (Sonoda et al., 2002). The estrogen-dependent MCF-7 cell growth will be evaluated using hemocytometer or ^{3}H-thymidine labeling method. We predict that activation of PXR induces EST expression, increase estrogen sulfation, and thus inhibits estrogen-dependent cell growth in the breast cancer MCF-7 cells.

KEY RESEARCH ACCOMPLISHMENTS

1. **The human EST promoter sequence has been cloned.** A 2034-bp promoter sequence (nt -2011 to nt +23) of the human EST promoter (hEST-p2kb) has been cloned by PCR using human placenta genomic DNA as the template. The PCR oligos are: 5' GTGCCAGCTTTACACTTGTTTTCAG 3' and 5' GATGAGAACCACTTCTGCATTTGGA 3'. The PCR products were subsequently cloned into pGL3 vector (Promega) upstream of luciferase reporter gene. The potential activation of hEST promoter by PXR is being investigated.

2. **The mouse EST promoter sequences have been cloned.** A 4210-bp promoter sequence (nt -4164 to nt +46) of the mouse EST promoter (mEST-p4.2kb) has been cloned by PCR using the bacterial artificial chromosome (BAC) clone RP24-571N6 (BACPAC Resource Center) as the template. The PCR oligos are: 5' TACCGCCTTTGGGATTGGTTTCCTTTTG 3' and 5' TGGCAGCACGATTCTTCAGCTCTG 3'. The PCR products were subsequently cloned into pGL3 vector upstream of luciferase reporter gene. A series of 5' deletions of the promoter were generated by PCR using mEST-p4.2kb as the template (Fig.1). The potential activation of mEST promoter and its deletion variants by PXR is being evaluated.

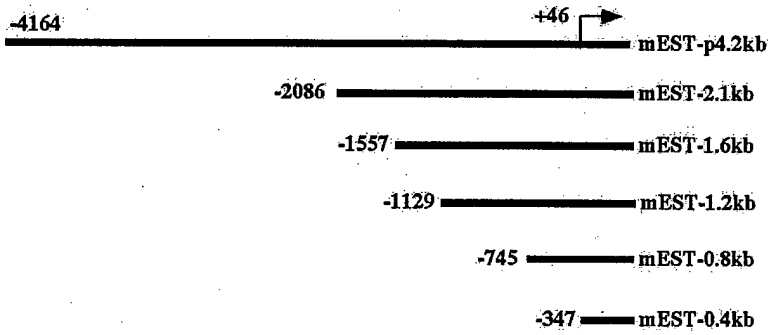

Fig.1. Schematic representations of the 4.2 kb mouse EST gene promoter and its 5' deletion mutants. The positions of nucleotides are indicated.

3. **Attempt to create PXR-expressing breast cancer MCF-7 cells.**

Methods: A retroviral transfection method was utilized to create PXR expressing breast cancer cells. Figure 2A depicts the strategy used to create stable cells. The cDNAs of

the wild type hPXR or activated VP-hPXR were cloned into the pBabe retroviral vector (Tontonoz et al., 1994). For retroviral production, pBabe-hPXR, pBabe-VP-hPXR, or the control pBabe-Puro constructs were transfected into Phoenix-Ampho helper-free retroviral producing cells using Lipofectamine 2000 (Invitrogen). The cell culture medium that contained the retrovirus was harvested 48 h after transfection and used to infect target MCF-7 cells plated on 10 cm tissue culture plates. Twenty four hours after infection, the cells were replaced with medium supplemented with puromycin (2-4 μg/ml). After selecting for 2-3 weeks, 6-10 individual clones were picked and the remaining clones were pooled and expanded in selection medium. To confirm the expression of the transduced hPXR mRNA, total RNA was prepared and subjected to Northern blot analysis using a hPXR cDNA probe.

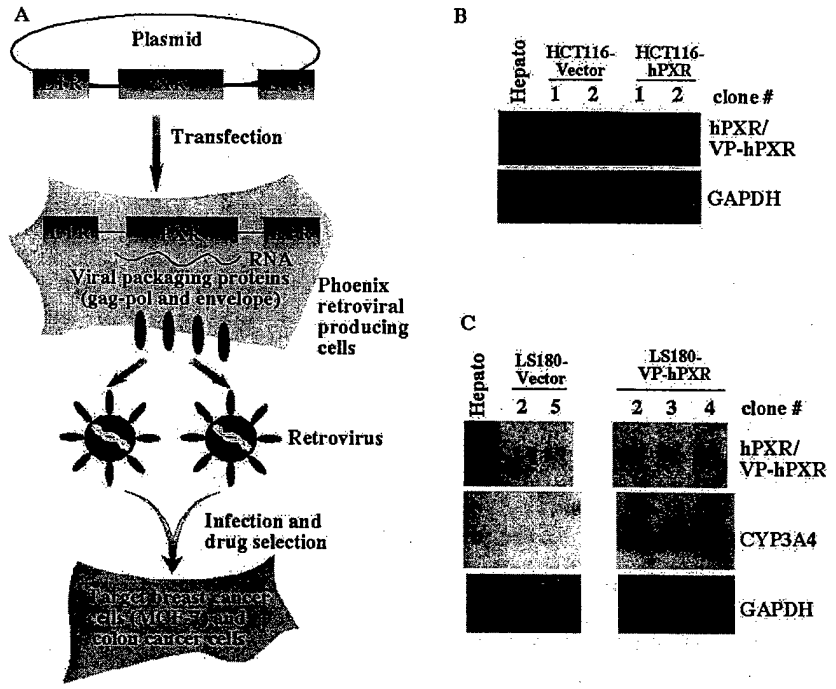

Fig.2. Creation of hPXR and VP-hPXR expressing cancer cell lines. (A) Schematic representation of the retroviral transfection method. The protocol includes the production of PXR-coding retroviruses and infection of target cancer cell lines; (B) The colon cancer HCT116 cells transfected with vector or hPXR The expression of hPXR mRNA was confirmed by Northern blot analysis. RNA derived from human hepatocytes (Hepato) was included as a positive control; (C) The colon cancer LS180 cells transfected with VP-hPXR showed constitutive induction of endogenous CYP3A4 mRNA expression.

Results: We failed to obtain PXR-expressing MCF-7 cells despite repeated effort. However, in the parallel experiments, we have successfully generated PXR-expressing colon cancer cells. As shown in Fig. 2B, the vector transfected colon cancer HCT116 cells exhibited minimal expression of hPXR as revealed by Northern blot. In contrast, the transduced hPXR mRNA was readily detectable in HCT116-hPXR cells. The wild type colon caner LS180 cells express low, but detectable, levels of hPXR. Expression of VP-hPXR caused a robust induction of endogenous CYP3A4 mRNA and the levels of CYP3A4 induction were correlated with the expression levels of VP-hPXR (Fig. 2C).

Future plan: We are in the process of developing another retroviral strategy in order to produce PXR-expressing MCF-7 cells. As diagramed in Figure 3, in addition to the hPXR cDNA, this retroviral vector will also contain a GFP marker that separated with the hPXR by an internal ribosomal entry site (IRES) (Liu et al., 2000). Because of the GFP marker, the infected MCF-7 cells can be sorted by flow cytometry and the GFP

containing cells, also the PXR expressing cells, will be enriched before plating and additional drug selection.

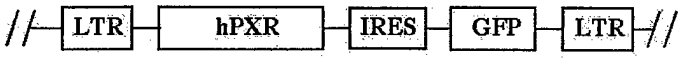

Fig.3. A retroviral vector that will allow GFP-mediated cell sorting. The cDNAs of hPXR and GFP were separated by an IRES site that will allow the expression of both hPXR and GFP in the transfected cells. The GFP will be used as a cell sorting marker.

4. Other project-related development

(1) Activation of PXR in transgenic mice induced estrogen sulfation. We have previously created transgenic mice that express the activated PXR (VP-PXR) in liver (Xie et al., 2000a). EST activity was evaluated by using liver cytosolic extractions of the wild type and transgenic mice. We used estrone as the sulfation substrate and ^{35}S-PAPS as the sulfate donor (Saini et al., 2004). Figure 4 shows that EST activity was significantly higher in VP-PXR mice as compared to the wild type mice.

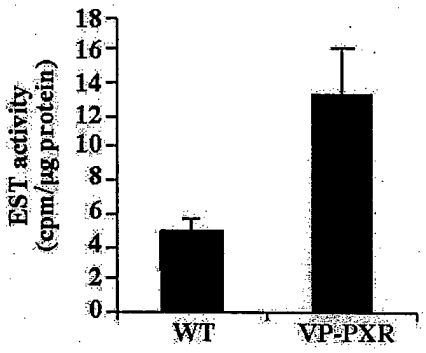

Fig.4. EST activity is increased in transgenic mice expressing the activated PXR (VP-PXR). The liver cytosolic extractions were measured for EST activity using estrone as the substrate.

(2) Sufotransferases (SULTs) are also regulated by the orphan nuclear receptor CAR.

(a) Identification of CAR binding sites within the SULT gene promoters. As an initial effort to examine whether the expression of SULTs is also under the control of constitutive androstane receptor (CAR), another so-called "xenobiotic receptor", we analyzed the 5' flanking region of SULT genes. Sequence analysis of the rat SULT2A gene promoters revealed an IR-0 (inverted repeats without a spacing nucleotide) type of NR response element (Fig. 5A) (Runge-Morris et al., 1999; Song et al., 2001; Sonoda et al., 2002). The rat 2A1/IR0 element was shown to bind to and mediate the transactivation by PXR (Sonoda et al., 2002) and FXR (Song et al., 2001). We examined whether this IR0 element can also bind to CAR. Electrophoretic mobility shift assays (EMSA) were used to determine the ability of CAR to

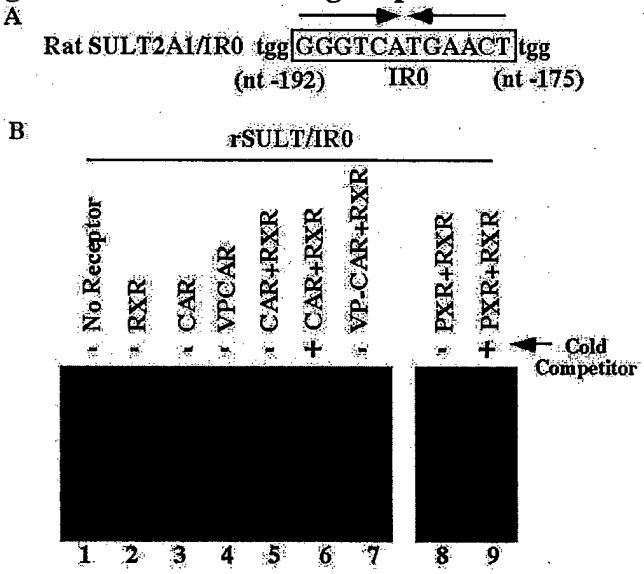

Fig.5. Identification of CAR binding site in SULT2A gene promoters. (A) The partial DNA sequence of the rat SULT2A1 gene promoter. The IR0 elements were boxed; (B) CAR/RXRα or VP-CAR/RXRα heterodimers bound to the IR0. EMSA was performed using *in vitro* synthesized receptor proteins and radiolabeled oligonucleotides. The binding of PXR/RXRα was included as a positive control.

bind to SULT/IR0 using *in vitro* synthesized receptor proteins and [^{32}P]-labeled oligonucleotide probe. As shown in Figure 5B, both the wild type CAR and its activated variant VP-CAR bound the rat SULT2A1/IR0 efficiently (lanes 5 and 7). The binding was dependent on the presence of their obligatory heterodimerization partner RXR; no DNA binding was seen in the absence of RXR (lanes 3 and 4). These results demonstrate that CAR/RXR or VP-CAR/RXR binds SULT/IR0 in a fashion similar to the binding of PXR/RXR to the same element (lane 8). The integrity of this IR0 element is essential for the binding, as the binding was abrogated when the IR0 was disrupted by mutation (data not shown). The binding of IR0 by CAR was also specific, in as much as efficient competition of binding was achieved by excess unlabeled wild type IR0 (Fig. 5B lane 6). Of note, while the VP-CAR exhibited a binding specificity similar to its wild type counterpart, VP-CAR appeared to have higher affinity toward the IR0 elements (Fig. 5B).

(b) CAR activates SULTs in cultured cells. Transfection based assays were utilized to determine whether CAR can transactivate SULT by binding to the IR0 elements in cultured cells. First, luciferase reporter genes, containing the wild type rat IR0 or its mutant variant upstream of a minimal thymidine kinase (tk) promoter, were constructed and transfected into CV-1 cells together with expression vectors for mouse CAR or PXR receptor in the presence of RXR. A panel of mCAR agonist and inverse agonist compounds were tested. As shown in Figure 6A, reporter genes derived from the rat SULT2A gene was activated by CAR in the absence of ligand. The activation was substantially inhibited by the inverse agonist androstenol but modestly potentiated by the agonist TCPOBOP. The agonistic effect of TCPOBOP was better manifested by its ability to reverse the inhibitory effect of androstenol when both ligands were added simultaneously, consistent with previously reports (Honkakoski et al., 1998; Tzameli et al., 2000 et al., Xie et al., 2000b). The activation by CAR was abrogated when the IR0

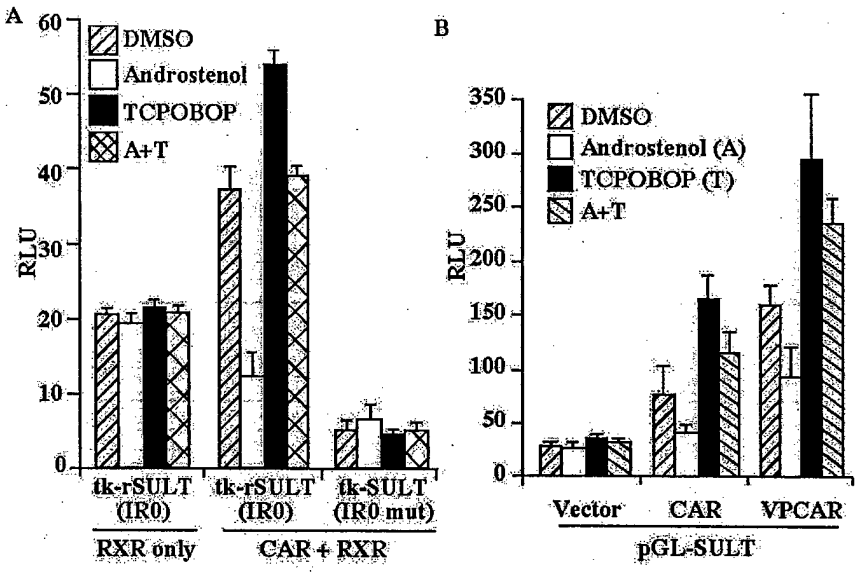

Fig.6. CAR activates SULT gene expression in cell cultures. (A) The synthetic tk-SULT/IR0-Luc reporters or its mutant variant were transfected into CV-1 cells in the presence of expression vectors for CAR and RXRα. Cells were subsequently treated with individual or combination of compounds. Results shown are normalized relative luciferase units, and represent the averages and standard error from triplicate assays. (B) CAR-mediated activation of the natural rat SULT2A1 gene promoter. The natural SULT promoter was transfected into HepG2 cells in the presence of expression vectors for CAR or VP-CAR. Cells were subsequently mock treated or treated with indicated compounds. Concentrations of ligands are androstenol, 5 μM; TCPOBOP, 250 nM; PCN, 10 μM.

was disrupted (Fig. 6A). Thus, the IR0 sites are mediators for both the binding and activation of SULT2A by CAR.

The activation of SULT2A by the wild type or constitutively activated CAR was also seen when a luciferase reporter that contains the natural promoter of rat SULT2A1 gene (nt -1023 to +38) was used. The reporter, PGL-SULT, was co-transfected with the wild type or activated mCAR into the human hepatoma HepG2 cells or primary rat hepatocytes followed by ligand treatment. HepG2 or hepatocytes were used since this promoter was not responsive in non-hepatocyte derived cells (data not shown). Consistent with the observations in CV-1 cells, activation of the natural SULT promoter by CAR in HepG2 cells was inhibited by androstenol. TCPOBOP not only activated CAR by itself but also reversed the inhibitory effect of androstenol (Fig. 6B). Interestingly, while exhibiting significantly higher constitutive activity, VP-CAR is also subjected to ligand effects similar to its wild type counterpart.

REPORTABLE OUTCOMES

Published Manuscripts:

Saini SPS, Sonoda J, Xu L, Toma D, Hirdesh H, Mu Y, Ren S, Moore DD, Evans RM, and Xie W. A novel CAR-mediated and CYP3A-independent pathway of bile acid detoxification. Mol. Pharmacol. 65, 292-300 (2004)

Gardner-Stephen D, Heydel JM, Goyal A, Lu Y, Xie W, Lindblom T, Mackenzie P, Radominska-Pandya A. Human PXR variants and their differential effects on the regulation of human UDP-glucuronosyltransferase gene expression. Drug Metabolism and Deposition 32, 340-347 (2004)

Xie W, Uppal H, Saini SPS, Mu Y, Little JM, Radominska-Pandya A, and Zemaitis MA. Orphan nuclear receptor-mediated xenobiotic regulation in drug metabolism and human diseases. Drug Discov. Today 9, 442-449 (2004).

Meeting Abstracts:

Saini SPS, Sonoda J, Xu L, Toma D, Hirdesh H, Mu Y, Ren S, Moore DD, Evans RM, and Xie W. A novel CAR-mediated and CYP3A-independent pathway of bile acid detoxification. *Nuclear Receptor: Orphan Brothers, Keystone Symposium*, Keystone Resort, Keystone, Colorado. February 28 - March 4, 2004.

Saini SPS, Sonoda J, Xu L, Toma D, Hirdesh H, Mu Y, Ren S, Moore DD, Evans RM, and Xie W. A novel CAR-mediated and CYP3A-independent pathway of bile acid detoxification. *University of Pittsburgh Cancer Institute Research Retreat*, 2003.

Supported Personnel:

Wen Xie, M.D., Ph.D., Principal Investigator (08/01/03 – 07/31/04)
Ying Mu, Ph.D., Postdoctoral Research Associated (08/01/03 – 11/30/03)
Haibiao Gong, Postdoctoral Research Associated (12/01/03 – 07/31/04)

CONCLUSIONS

We have successfully cloned the human and rodent EST gene promoters. The EST promoter will be evaluated for PXR activation by transfection and reporter gene assays. Although we were able to create PXR-expressing colon cancer cells, we have yet to create stable PXR-expressing MCF-7 cells that will be used to evaluate the PXR-mediated EST activation and E2 deprivation in breast cancer cells. A new retroviral transfection system is being tested to overcome the difficulty of obtaining PXR-expressing MCF-7 cells. In several related development, we have shown that mice expressing the activated PXR in the liver had increase EST activity, further supporting our hypothesis that EST is regulated by PXR. Our observation that sulfotransferases are also under the transcriptional control of CAR suggests a coordinate regulation of sulfotransfeases, include EST, by xenobiotic orphan receptors that included PXR and CAR. It is hoped that activation of PXR will represent a novel strategy to functionally deprive estrogen activity and to treat and to prevent breast cancers. The results generated from this DOD funded research project are expected to be used to apply for a more comprehensive grant application on breast cancer chemoprevention.

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APPENDICES (ACKNOWLEDGED AND RELATED PUBLICATIONS)

Saini SPS, Sonoda J, Xu L, Toma D, Hirdesh H, Mu Y, Ren S, Moore DD, Evans RM, and Xie W. A novel CAR-mediated and CYP3A-independent pathway of bile acid detoxification. Mol. Pharmacol. 65, 292-300 (2004)

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A Novel Constitutive Androstane Receptor-Mediated and CYP3A-Independent Pathway of Bile Acid Detoxification

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ABSTRACT

Cytosolic sulfotransferase (SULT)-mediated sulfation plays an essential role in the detoxification of bile acids and is necessary to avoid pathological conditions, such as cholestasis, liver damage, and colon cancer. In this study, using transgenic mice bearing conditional expression of the activated constitutive androstane receptor (CAR), we demonstrate that activation of CAR is both necessary and sufficient to confer resistance to the hepatotoxicity of lithocholic acid (LCA). Surprisingly, the CAR-mediated protection is not attributable to the expected and previously characterized CYP3A pathway; rather, it is associated with a robust induction of SULT gene expression and increased LCA sulfation. We have also provided direct evidence that CAR regulates SULT expression by binding to the CAR response elements found within the SULT gene promoters. Interestingly, activation of CAR was also associated with an increased expression of the 3′-phosphoadenosine 5′-phosphosulfate synthetase 2 (PAPSS2), an enzyme responsible for generating the sulfate donor 3′-phosphoadenosine-5′-phosphosulfate. Analysis of gene knockout mice revealed that CAR is also indispensable for ligand-dependent activation of SULT and PAPSS2 in vivo. Therefore, we establish an essential and unique role of CAR in controlling the mammalian sulfation system and its implication in the detoxification of bile acids.

Bile acids are end products of cholesterol catabolism that function as both a detergent to solubilize circulating cholesterol remnants and lipophilic vitamins and as a signaling molecule to regulate its own homeostasis. When bound to and activated by bile acids, the farnesoid X receptor (FXR) represses transcription of cholesterol 7α-hydroxylase (CYP7A), the rate-limiting enzyme of bile acid synthesis, thereby repressing the conversion of cholesterol to bile acids (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). Two other hepatic factors, the small heterodimer partner and liver receptor homolog-1 were subsequently found to be involved in the FXR-mediated CYP7A repression (Goodwin et al., 2000; Lu et al., 2000). In addition to their beneficial function, excessive bile acids are potentially toxic when accumulated. For example, the secondary bile acid LCA is a potent cholestatic agent and can cause histological liver damage and other pathological changes unless it is efficiently eliminated (Leuschner et al., 1977). Several lines of evidence have also suggested that toxic bile acids can function as tumor promoters to promote colon cancers (Narisawa et al., 1974).

The efficient detoxification and clearance of bile acids requires the phase I CYP3A enzymes and the phase II cytosolic sulfotransferases (SULTs). The CYP3A enzymes catalyze the hydroxylation of LCA, which promotes LCA elimination (Staudinger et al., 2001; Xie et al., 2001). Recently, the activation of the pregnane X receptor (PXR) and vitamin D receptor (VDR) and subsequent induction of CYP3A enzyme has been proposed to be a means to eliminate toxic bile acids (Staudinger et al., 2001; Xie et al., 2001; Makishima et al.,

This work was supported in part by National Institutes of Health grant R01-ES12479 (to W.X.), the Competitive Medical Research Fund and the Central Research Development Fund from the University of Pittsburgh (to W.X.), and the National Institutes of Health Nuclear Receptor Signaling Atlas orphan receptor program grant U19-DK62434-01 (to R.M.E.). R.M.E. is an Investigator of the Howard Hughes Medical Institute and March of Dimes Chair in Molecular and Developmental Biology.

ABBREVIATIONS: FXR, farnesoid X receptor; LCA, lithocholic acid; SULT, cytosolic sulfotransferase; PXR, pregnane X receptor; CAR, constitutive androstane receptor; VDR, vitamin D receptor; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PB, phenobarbital; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; PAPSS2, 3′-phosphoadenosine 5′-phosphosulfate synthetase 2; VP, virus protein; EMSA, electrophoretic mobility shift assay; RXR, retinoid X receptor; DHEA, dehydroepiandrosterone; TetRE, tetracycline responsive element; tTA, tetracycline-responsive transcriptional activator; Dox, doxycycline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

2002). The phase II SULTs are also important for bile acid detoxification. SULTs catalyze the transfer of a sulfonyl group from the cosubstrate 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to the acceptor substrates to form sulfate or sulfamate conjugates. LCA is a preferred substrate for SULT2A9/hydroxysteroid sulfotransferase (Chen and Segel, 1985; Radominska et al., 1990; Song et al., 2001). The sulfated LCA shows less cytotoxicity than LCA when exposed to cells or animals (Leuschner et al., 1977). Although SULTs play a key role in a number of critical biological pathways, little is known about the regulatory pathways that control SULT gene expression.

The orphan nuclear constitutive androstane receptor (CAR) was first shown to function as a xenobiotic receptor by activating the CYP2B genes. Subsequently, this activation was found to be potentiated by phenobarbital (PB) and TCPOBOP (Honkakoski et al., 1998; Tzameli et al., 2000; Xie et al., 2000a), and the latter was identified as a CAR agonistic ligand. Response to both inducers was completely lost in CAR knockout mice (Wei et al., 2000). CAR was later shown to cross-regulate *CYP3A* genes in cell cultures by sharing the previously identified PXR response elements (Xie et al., 2000b; Goodwin et al., 2001; Wei et al., 2002). CAR was more recently implicated in transactivating genes that encode the phase II UDP-glucuronosyltransferase 1A1 (Sugatani et al., 2001; Huang et al., 2003; Xie et al., 2003) and the drug transporter multidrug resistance-associated protein 2 (MRP2) (Kast et al., 2002). Although CAR has been well characterized as a cytochrome P450 gene regulator, no direct evidence that relates the function of this xenobiotic receptor to the transcriptional activation of SULT is available. PB and TCPOBOP have been shown to induce members of the SULT family, although the molecular basis remains to be defined (Runge-Morris et al., 1999; Garcia-Allan et al., 2000; Maglich et al., 2002). Both PB and TCPOBOP are efficacious CAR activators, suggesting this as a plausible signaling pathway for SULT transcription. The SULT induction by TCPOBOP is apparently CAR-dependent (Maglich et al., 2002), but a DNA microarray analysis with PB-treated mice failed to identify SULT as a target (Ueda et al., 2002).

In this report, we show that activation of CAR in transgenic mice confers resistance to the hepatotoxicity of LCA. The protection is not caused by CYP3A; instead, it is associated with the induction of both SULTs and PAPS synthetase 2 (PAPSS2), an enzyme responsible for generating the cosubstrate PAPS. Moreover, CAR is indispensable for SULT and PAPSS2 induction by PB and TCPOBOP. We propose that activation of CAR facilitates bile acid detoxification via a combined induction of the sulfation system.

Materials and Methods

Animals, Drug Treatment, and Histology Evaluation. The creation of PXR and CAR null mice has been described before (Wei et al., 2000; Xie et al., 2000b). The PXR/CAR double-knockout mice were created by cross-breeding. When necessary, mice were subjected to a single intraperitoneal injection of PB (40 mg/kg) or TCPOBOP (3 mg/kg) 24 h before sacrifice. To generate the tetracycline responsive element (TetRE)VP-CAR transgene, VP-CAR cDNA was excised from pCMX-VP-mCAR (Xie et al., 2000a), and cloned into the TetRE transgene cassette (Xie et al., 1999). The Lap-tTA mice were obtained from The Jackson Laboratory (Bar Harbor, ME). When necessary, doxycycline (Dox; Sigma, St. Louis, MO) was diluted in 5% sucrose in water to a final concentration of 2 mg/ml and supplied as drinking water. The Dox-laced water was changed every 2 to 3 days. For LCA treatment, mice were given daily treatments of LCA (8 mg/day) or vehicle via gavage and were sacrificed 24 h after the last treatment (Xie et al., 2001). For histology evaluation, tissues were fixed in 4% formaldehyde, embedded in paraffin, sectioned at 5 μm, and stained for hematoxylin and eosin. The use of mice in this study has complied with all relevant federal guidelines and institutional policies.

DNA-Binding Analysis. Electrophoretic mobility shift assays (EMSA) were performed using in vitro-transcribed and -translated proteins (TNT; Promega, Madison, WI) as described previously (Xie et al., 2000a). Oligonucleotides used were: rat SULT2A1/IR0, 5′-TTTGGGGGTCATGAACTTGGGC-3′; mouse SULT2A9/IR0, 5′-TTTGG GGGTAATGAACT TGGGC-3′; and SULT/IR0 mut, 5′-TTTGGGGGTACCGAACTTGGGC-3′.

Plasmid Constructs and Transfection. The synthetic reporter thymidine kinase (tk)-IR0-Luc, the natural promoter reporter pGL-SULT, and their mutant variants were described before (Sonoda et al., 2002). The expression vectors for mCAR, hPXR, mPXR, and hRXRα were as described previously (Xie et al., 2000a,b). CV-1 and HepG2 cells were transfected in 48-well plates using *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (Roche, Indianapolis, IN) and LipofectAMINE (Invitrogen, Carlsbad, CA), respectively. When necessary, cells were treated with androstenol (5 μM), TCPOBOP (250 nM), pregnenolone-16α-carbonitrile (10 μM), and St. John's wort (300 μg/ml) in media containing 10% charcoal-stripped serum. The transfection efficiency was normalized against the β-galactosidase activities from the cotransfected CMX-β-galactosidase vector.

Northern Blot Analysis. Total RNAs were prepared from tissues using the TRIzol Reagent (Invitrogen). Northern hybridization was carried out as described previously (Xie et al., 2000b). The cDNA probes for SULTs, PAPSS2, CYP3A11, and CYP2B have been described previously (Xie et al., 2000a, b; Sonoda et al., 2002).

Sulfotransferase Assay. Sulfotransferase assay was carried out using [^{35}S]PAPS (PerkinElmer Life and Analytical Sciences, Boston, MA) as described previously (Sonoda et al., 2002). In brief, 5 to 10 μg/ml total liver cytosolic extract was used with 2 μM LCA, 5 μM dehydroepiandrosterone (DHEA), or 4 μM *p*-nitrophenol as substrate. After the reactions, free [^{35}S]PAPS was removed by extracting with ethyl acetate. The aqueous phase was then measured in a liquid scintillation counter for radioactivity. Control reactions that do not contain substrate were also carried out in parallel, and their radioactivity was subtracted from test reactions. Two to four pairs of mice were used for each SULT assay, and each reaction was run in triplicate.

Results

Conditional Expression of the Activated CAR in Transgenic Mice. To examine the effects of CAR activation in xenobiotic regulation, we created a transgenic mouse system that allowed conditional expression of a constitutively activated CAR (VP-CAR) (Xie et al., 2000a) in the liver. Two lineages of transgenic mice were used as diagramed in Fig. 1A. First, we created the TetRE-VP-CAR transgene that encodes VP-CAR under the control of a minimal cytomegalovirus promoter and the TetRE (Fig. 1A). The TetRE-VPCAR mice were subsequently bred with the Lap-tTA activator line to generate bi-transgenic animals. Driven by the liver-specific Lap (CCAAT/enhancer-binding protein-β) promoter, the Lap-tTA transgene directed the expression of the tetracycline-responsive transcriptional activator (tTA) constitutively and exclusively in the hepatocytes (Kistner et al., 1996). We anticipated that tTA bound to TetRE and conse-

quently induced the expression of VP-CAR only in the absence of Dox. Addition of Dox will result in the displacement of tTA from TetRE and will silence VP-CAR expression (Tet-Off).

Transgene expression was assessed by Northern blot analysis of liver RNA using the tTA cDNA probe. Because tTA contains the VP16 activation domain that is also present in VP-CAR, this probe recognizes mRNA of both tTA and VP-CAR. Similar levels of tTA expression were detected in the livers of all Lap-tTA transgenic mice whether they harbored this transgene alone or in combination with the TetRE-VP-CAR transgene (Fig. 1B). Moreover, expression of the Lap-tTA mRNA was independent of Dox treatment (Fig. 1B, compare lanes 4 and 5). Two tTA-specific transcripts were detected, consistent with our previous observation (Xie et al., 1999). No VP-CAR expression was detected in the TetRE-VP-CAR single transgenic mice (Fig. 1B, lane 2). In contrast, a robust expression of the 2.5-kilobase VP-CAR was achieved in the bitransgenic animal in the absence of Dox (Fig. 1B, lane 4). Moreover, the expression of CYP2B10, a known CAR target gene, was also markedly induced in the VP-CAR–expressing livers. As expected, the administration of Dox in drinking water in bitransgenics resulted in the silencing of both VP-CAR expression and CYP2B10 induction (Fig. 1B, lane 5). Therefore, the expression of VP-CAR in the liver was completely reversible upon Dox treatment. The expression of VP-CAR was restricted to the liver. No VP-CAR transcripts were detected in the intestine, and tTA was not expressed in the intestine (data not shown). The hepatic expression of the endogenous mouse CAR remained unchanged in the presence of VP-CAR (data not shown). Because the presence of single transgene did not cause VP-CAR expression and had no effect on the expression of a number of known CAR targets genes that we examined (Fig. 1B; data not shown), both the single transgenic and wild-type mice were used as control animals for the bitransgenic mice in the following animal experiments.

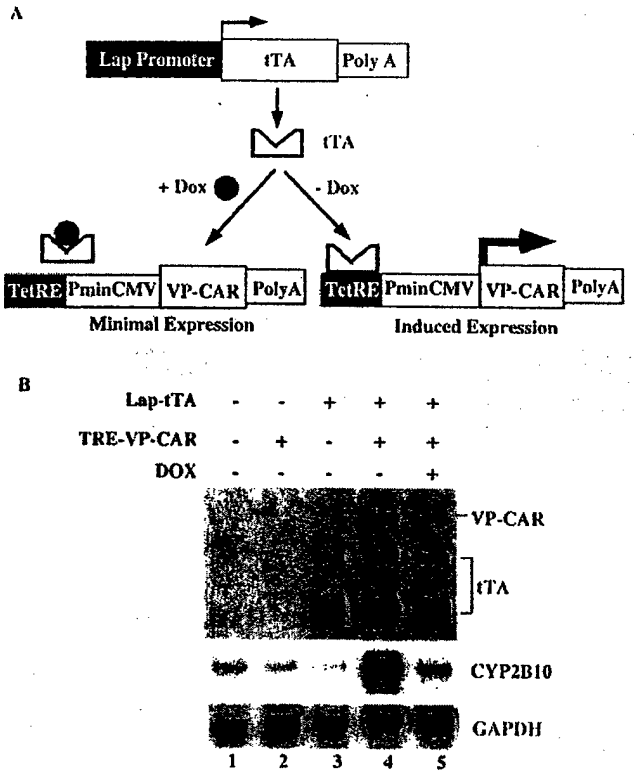

Fig. 1. Creation of transgenic mice that harbor conditional expression of the activated CAR in the liver. A, a schematic outline of the Lap-tTA/TetRE-VP-CAR two-component Tet-Off transgenic system. The Lap-tTA transgene directs the expression of the tTA activator to the liver. The binding of tTA to the TetRE and the induction of the transgene VP-CAR should only occur in the absence of Dox. B, liver-specific conditional expression of VP-CAR. Liver RNAs of mice with indicated genotypes were subjected to Northern blot analysis. The mouse in lane 5 was subjected to 5 days of Dox treatment. The membrane was hybridized with the tTA probe that recognizes both tTA and VP-CAR transcripts. The membrane was subsequently stripped and reprobed with CYP2B10, with GAPDH as a loading control.

Activation of CAR Confers Resistance to LCA Hepatotoxicity. The secondary bile acid LCA is toxic and known to cause cholestasis and associated hepatotoxicity (Staudinger et al., 2001; Xie et al., 2001). To examine whether or not activation of CAR had effects on LCA sensitivity, adult bitransgenic mice or control littermates were dosed with vehicle solvent or LCA for 4 days before liver histological evaluation. As expected, the wild-type liver exhibited areas of necrosis after LCA exposure (Fig. 2B), consistent with our previous report (Xie et al., 2001). In a sharp contrast, the liver of bi-transgenic mice showed virtually no histological changes upon LCA treatment (Fig. 2C). The absence of induced pathology in the bi-transgenic mice demonstrates that sustained activation of CAR is sufficient to prevent LCA-mediated histological liver damage. This xenoprotection is CAR activation-dependent, because treatment of Dox blocked protection (Fig. 2D). As expected, Dox treatment alone had no effect on liver histology of the wild-type mice (data not shown), and the Dox-treated wild-type animals remained sensitive to LCA (Fig. 2E).

Activation of CAR Induces SULTs and PAPSS2 in Transgenic Mice. To delineate the molecular mechanism of LCA resistance, we profiled the expression of hepatic genes encoding bile acid-detoxifying enzymes in the VP-CAR mice. The phase I *CYP3A11*, a primary PXR target gene, has been shown to be cross-regulated by CAR in cell cultures (Xie et al., 2000a). Much to our surprise, the expression of CYP3A11 mRNA was slightly suppressed rather than induced in the bitransgenic mice (Fig. 3A). A CYP3A enzymatic assay using testosterone as a substrate also confirmed the absence of CYP3A induction in VP-CAR mice (data not shown). In contrast, the expression of the phase II SULT 2A9 mRNA was markedly induced in the VP-CAR mice (Fig. 3A). The induction was seen in both sexes, although the female mice had a higher basal level of this isoform (Klaassen et al., 1998). The sustained induction of SULT2A9 was VP-CAR dependent, because treatment with Dox for 7 days resulted in complete loss of 2A9 induction in both male and female mice, presumably because of the absence of VP-CAR expression (Fig. 1B). As expected, Dox treatment has no effect on the expression either SULT2A9 or CYP3A11 in the control mice (Fig. 3A). Of note was that the expression of CYP3A11 in the VP-CAR mice remained inducible in response to TOPOBOP (Fig. 3B), suggesting that the lack of CYP3A11 induction in the unchallenged mice was not caused by the unresponsiveness of CYP3A11 in this transgenic line.

We also analyzed the expression of several other SULT isoforms. The expression of SULT1A4 was increased in the

bitransgenic mice, whereas the expression of SULT1D1 remained unchanged (Fig. 3C). Thus, the regulation of SULT by CAR seemed to be isoform-specific. The sulfation reaction requires the donation of a sulfonyl group from the cosubstrate PAPS. Surprisingly, the expression of hepatic PAPSS2, the enzyme that catalyzes the formation of PAPS from inorganic sulfate (Lyle et al., 1994), was also elevated in the bitransgenic mice (Fig. 3D, lane 2). The PAPSS2 induc-

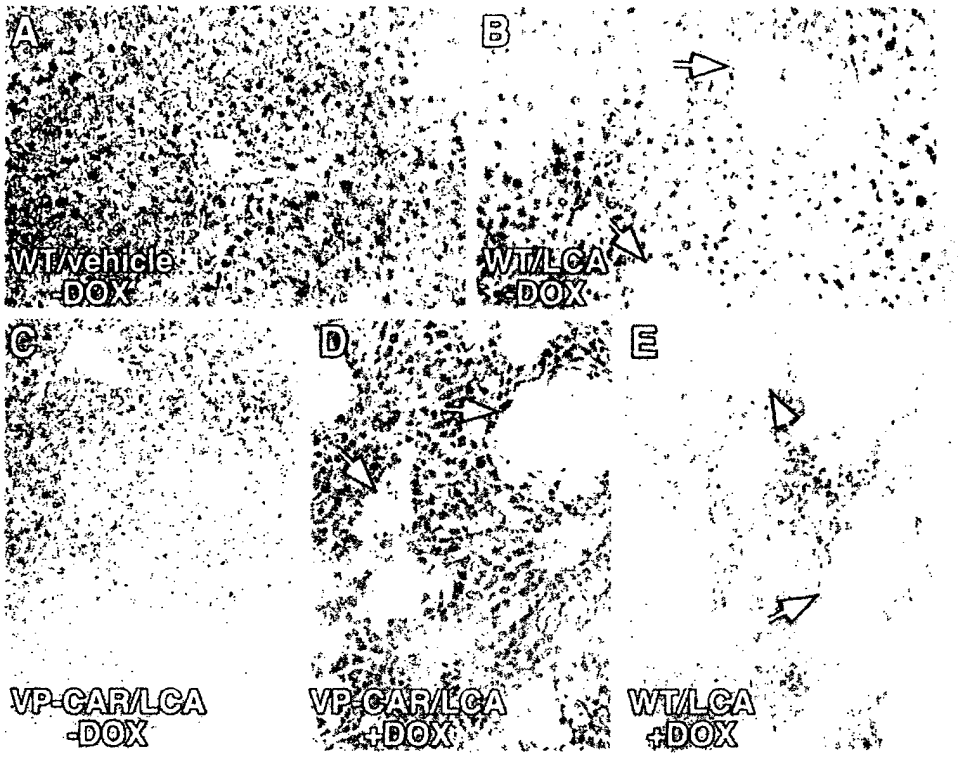

Fig. 2. Activation of CAR confers resistance to LCA-induced hepatotoxicity. Results shown are liver paraffin sections stained with hematoxylin and eosin. Mice of indicated genotypes were given daily treatments of vehicle (A) or LCA (B–E) for 4 days. Mice in D and E were treated with Dox water for 5 days before LCA exposure, and Dox treatment continued during LCA treatment. Regions of liver necrosis are marked by arrows. Magnification, 200×.

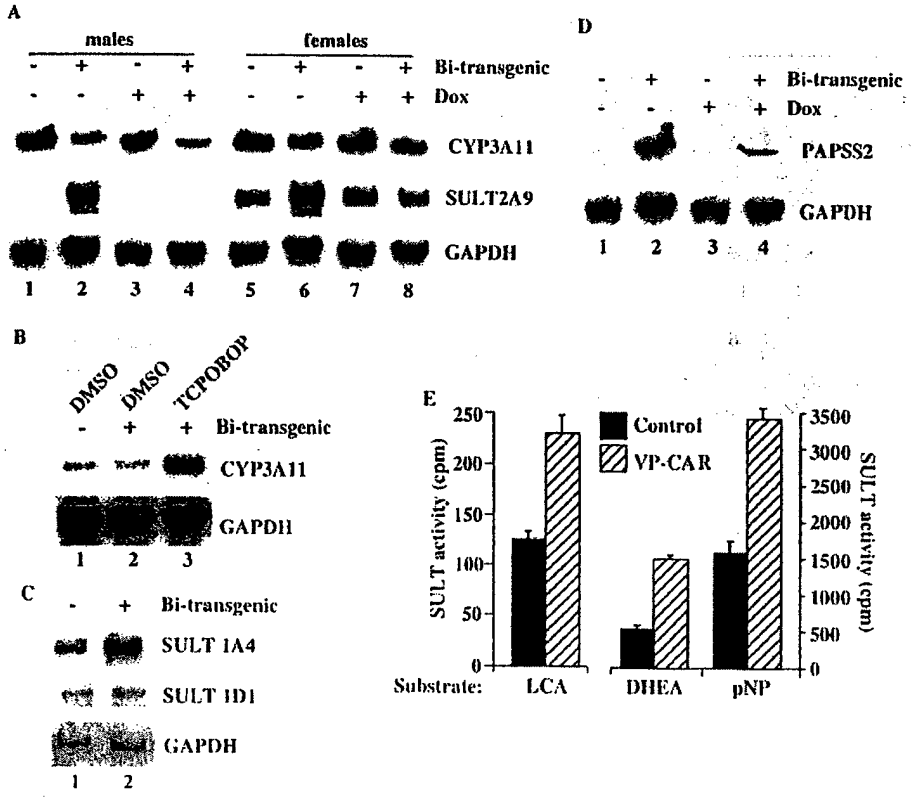

Fig. 3. The expression of SULTs, but not CYP3A11, is induced in the VP-CAR mice. A–C, total liver RNAs were subjected to Northern blot analysis. The membranes were probed for CYP3A11 and SULT2A9 (A), CYP3A11 (B), SULT1A4 and 1D1 (C), and PAPSS2 (D). Mice in lanes 3, 4, 7, and 8 of A and lanes 3 and 4 of D were treated with Dox for 7 days before tissue harvest. GAPDH probing was used as loading control. E, increased SULT activity in VP-CAR mice. Cytosolic liver extracts from bitransgenic male mice or control littermates were subjected to sulfation assay using the substrates of LCA, DHEA, and *p*-nitrophenol. [^{35}S]PAPS was used the sulfate donor. Radioactivity was measured by scintillation counter after separation and removal of free [^{35}S]PAPS. Results represent the averages and standard error.

tion was lost upon 7 days of Dox treatment (Fig. 3D, lane 4). Our data suggest that CAR may function as a global regulator of sulfation cascade by controlling the production of both SULT enzymes and the cosubstrate PAPS.

The increased expression of SULTs was also reflected at enzymatic levels. SULT2A9 is known to sulfonate bile acids such as LCA, steroid hormones and their precursors, such as DHEA, and carcinogenic xenobiotics, such as *p*-nitrophenol. Compared with the control mice, the bitransgenic animals exhibited about 2-fold higher hepatic sulfation activity toward LCA (Fig. 3E). The sulfation of DHEA and *p*-nitrophenol was also more than doubled (Fig. 3E). Together, these results suggest that members of the cytosolic SULT family are under the positive control of CAR.

Identification of CAR Binding Sites within the SULT Gene Promoters. To understand the underlying mechanism of *SULT* regulation by CAR, we went on to analyze the 5′ flanking region of *SULT* genes. Sequence analysis of the rodent *SULT2A* gene promoters revealed an IR-0 (inverted repeats without a spacing nucleotide) type of nuclear receptor response element (Fig. 4A) (Runge-Morris et al., 1999; Song et al., 2001; Sonoda et al., 2002). The rat 2A1/IR0 element was shown to bind to and mediate the transactivation by PXR (Sonoda et al., 2002) and FXR (Song et al., 2001).

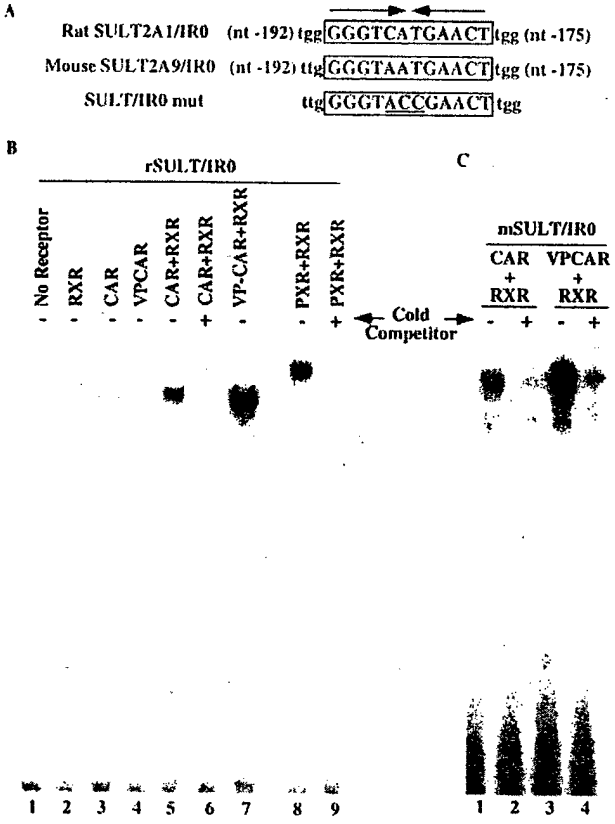

Fig. 4. Identification of CAR binding sites in the rodent SULT gene promoters. A, the partial DNA sequence of the rat *SULT2A1* and mouse *SULT2A9* gene promoters. The IR0 elements are boxed. A mutant variant was also shown with the mutated nucleotides underlined. B and C, CAR/RXRα or VP-CAR/RXRα heterodimers bound to the IR0. EMSA was performed using in vitro-synthesized receptor proteins and radiolabeled oligonucleotides of rat (B) or mouse (C) IR0. The binding of PXR/RXRα was included as a positive control.

We examined whether this IR0 element can also bind to CAR. EMSAs were used to determine the ability of CAR to bind to SULT/IR0 using in vitro-synthesized receptor proteins and ^{32}P-labeled oligonucleotide probe. As shown in Fig. 4B, both the wild-type CAR and its activated variant VP-CAR bound the rat SULT2A1/IR0 efficiently (Fig. 4B, lanes 5 and 7). The binding was dependent on the presence of their obligatory heterodimerization partner RXR; no DNA binding was seen in the absence of RXR (Fig. 4B, lanes 3 and 4). These results demonstrate that CAR/RXR or VP-CAR/RXR binds SULT/IR0 in a fashion similar to the binding of PXR/RXR to the same element (Fig. 4B, lane 8). This represents another example of the sharing of binding sites by xenobiotic nuclear receptors (Xie et al., 2000a). The integrity of this IR0 element is essential for the binding, because the binding was abrogated when the IR0 was disrupted by mutation (data not shown). The binding of IR0 by CAR was also specific, inasmuch as efficient competition of binding was achieved by excess unlabeled wild-type IR0 (Fig. 4B, lane 6). Specific binding of both CAR and VP-CAR to the conserved mouse SULT2A9/IR0 was also observed (Fig. 4C). Whereas the VP-CAR exhibited a binding specificity similar to that of its wild-type counterpart, VP-CAR seemed to have higher affinity toward the IR0 elements (Fig. 4, B and C).

CAR Activates SULTs in Cultured Cells. Transfection-based assays were used to determine whether CAR can transactivate *SULT* by binding to the IR0 elements in cultured cells. First, luciferase reporter genes, containing the wild-type rat and mouse IR0 or their mutant variant upstream of a minimal tk promoter, were constructed and transfected into CV-1 cells together with expression vectors for mouse CAR or PXR receptor in the presence of RXR. A panel of mCAR agonist and inverse agonist compounds was tested. As shown in Fig. 5A, reporter genes derived from both rat and mouse *SULT2A* genes were activated by CAR in the absence of ligand. The activation was substantially inhibited by the inverse agonist androstenol but modestly potentiated by the agonist TCPOBOP. The agonistic effect of TCPOBOP was better manifested by its ability to reverse the inhibitory effect of androstenol when both ligands were added simultaneously, consistent with previously reports (Honkakoski et al., 1998; Tzameli et al., 2000 et al., Xie et al., 2000b). As expected, PXR also activated the same reporter genes (Sonoda et al., 2002) but with a distinctive ligand profile (Fig. 5B). For example, androstenol only showed marginal effect, whereas TCPOBOP is completely ineffective on PXR. In contrast, St. John's wort, an herbal antidepressant and PXR activator, induced SULT reporter genes by activating PXR (Fig. 5B) but not CAR (data not shown). Consistent with DNA binding results, the activation by CAR or PXR was abrogated when the IR0 was disrupted (Fig. 5, A and B). Thus, the IR0 sites are mediators for both the binding and activation of SULT2A by CAR.

The activation of SULT2A by the wild-type or constitutively activated CAR was also seen when a luciferase reporter that contains the natural promoter of rat SULT2A1 gene (nucleotides −1023 to +38) was used. The reporter, PGL-SULT, was cotransfected with the wild-type or activated mCAR into human hepatoma HepG2 cells or primary rat hepatocytes followed by ligand treatment. HepG2 or hepatocytes were used because this promoter was not responsive in non–hepatocyte-derived cells (data not shown). Con-

sistent with the observations in CV-1 cells, activation of the natural SULT promoter by CAR in HepG2 cells was inhibited by androstenol. TCPOBOP not only activated CAR by itself but also reversed the inhibitory effect of androstenol (Fig. 5C, lane 2). Interestingly, although VP-CAR exhibited significantly higher constitutive activity, it was also subjected to ligand effects similar to those of its wild-type counterpart (Fig. 5C, lane 3). The activation by CAR and VP-CAR was abolished when a promoter variant that contains the mutant IR0 was cotransfected (lanes 4 and 5).

Car Is Indispensable for Ligand-Dependent Activation of SULT. The expression of rodent SULT has been shown to be induced by PB and TCPOBOP, two reported CAR agonists (Runge-Morris et al., 1999; Garcia-Allan et al., 2000; Maglich et al., 2002). Having established that activation of CAR is sufficient to induce SULT2A9, we went on to examine whether xenobiotic receptors, such as CAR and PXR, are necessary for the ligand-dependent activation of SULT. We applied a single dose of PB and TCPOBOP to wild-type, PXR-null (Xie et al., 2000b), CAR-null (Wei et al., 2000), and PXR/CAR double-knockout mice. The double-knockout mice were created by cross-breeding, and the absence of both PXR and CAR mRNA was confirmed by Northern blot analysis (Fig. 6A). Livers were harvested 24 h after treatment, and the expression of SULT was evaluated by Northern blot analysis. As shown in Fig. 6B, the expression of both SULT2A9 and PAPSS2 was induced by either PB or TCPOBOP, as expected. Both the basal and PB- and TCPOBOP-inducible expression of SULT2A9 and PAPSS2 was sustained in the PXR-null mice, suggesting that PXR was dispensable for this induction. In contrast, disruption of the CAR locus led to a loss of SULT2A9 and PAPSS2 induction by TCPOBOP (Fig. 6C) and PB (data not shown) in both CAR-null and CAR/PXR double-knockout backgrounds. Together, our results demonstrate that CAR, but not PXR, is the bona fide receptor to mediate SULT and PAPSS2 induction by PB and TCPOBOP in vivo.

Discussion

Activation of PXR and VDR have been implicated in bile acid detoxification by inducing CYP3A (Staudinger et al., 2001; Xie et al., 2001; Makishima et al., 2002), but little is known about whether CAR also plays a role in bile acid detoxification. In this report, we show that the activation of CAR is both necessary and sufficient to confer resistant to the hepatotoxic LCA. Unexpectedly, the protection is CYP3A-

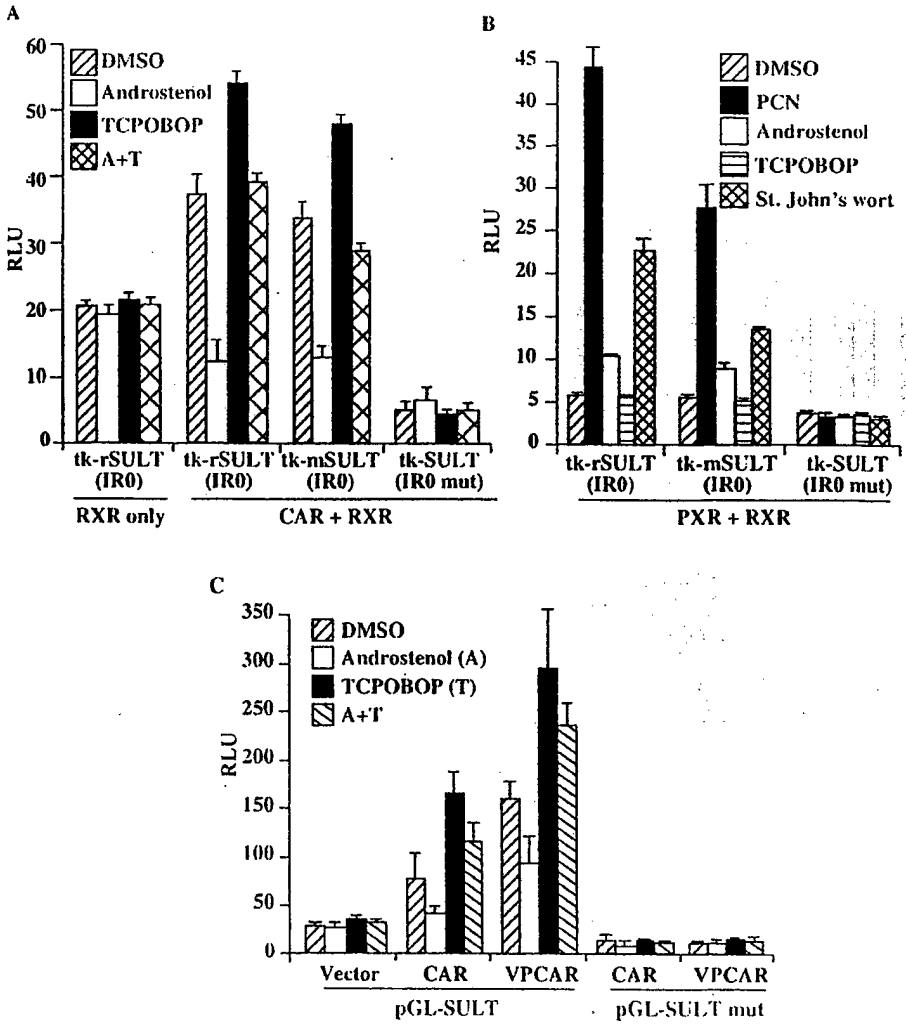

Fig. 5. CAR activates *SULT* gene expression in cell cultures. A, the synthetic tk-SULT/IR0-Luc reporters or their mutant variants were transfected into CV-1 cells in the presence of expression vectors for CAR and RXRα. Cells were subsequently treated with individual or combination of compounds. Results shown are normalized relative luciferase units and represent the averages and standard error from triplicate assays. B, similar transfections but using PXR receptor. C, CAR-mediated and IR0-dependent activation of the natural rat SULT2A1 gene promoter. The natural SULT promoter or its mutant variant were transfected into HepG2 cells in the presence of expression vectors for CAR or VP-CAR. Cells were subsequently mock-treated or treated with indicated compounds. Ligand concentrations: androstenol, 5 μM; TCPOBOP, 250 nM; pregnenolone-16α-carbonitrile (PCN), 10 μM; St. John's wort, 300 μg/ml.

independent, but can be explained, at least in part, by the ability of CAR to activate the detoxifying sulfonation system.

The identification of SULTs as targets of CAR has implications in bile acid detoxification, drug metabolism, and carcinogenesis. Sulfation is an essential step in the detoxification of bile acids and is necessary to avoid pathologic conditions, such as cholestasis, liver damage, and colon cancer (Fisher et al., 1971; Narisawa et al., 1974; Leuschner et al., 1977). The protection against LCA toxicity in VP-CAR mice suggests a potential therapeutic strategy for the design of CAR agonists to target cholestasis and to prevent colon cancer. We have previously shown that the PXR- and VDR-mediated CYP3A induction was also important for LCA clearance (Xie et al., 2001; Makishima et al., 2002). However, the resistance to LCA toxicity in the VP-CAR mice was clearly CYP3A-independent, because this enzyme was not induced in the VP-CAR mice (Fig. 3A). The attribution of SULT2A9 induction to the LCA resistance in VP-CAR mice is also consistent with a recent report that SULT2A9/hydroxysteroid sulfotransferase-mediated LCA sulfation was a major pathway for protection against LCA-induced hepatotoxicity. The FXR-null female mice exhibited enhanced resistance to LCA, which was associated with significantly increased hepatic SULT2A expression and LCA sulfation (Kitada et al., 2003). The excretion and elimination of bile acids are also facilitated by the UDP-glucuronosyltransferase (Radominska et al., 1990) and the canalicular bile acid transporter MRP2 (Kullak-Ublick et al., 2000); both are known CAR target genes (Sugatani et al., 2001; Kast et al., 2002; Huang et al., 2003; Xie et al., 2003), so we can not exclude the possibility that additional elements of the bile acid detoxifying system also contribute to the protection.

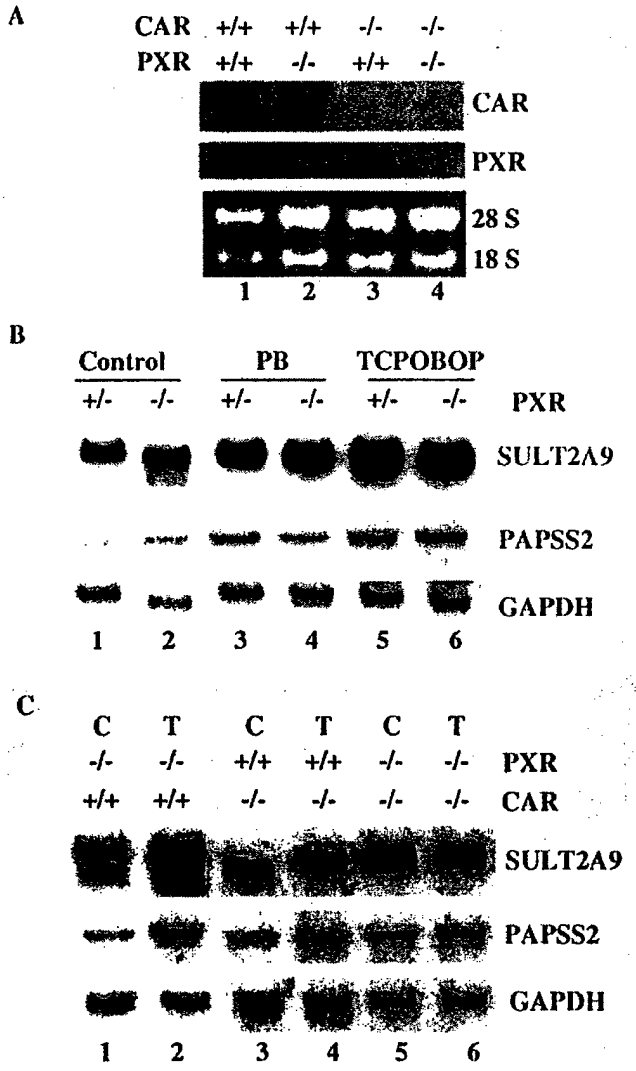

Fig. 6. CAR, but not PXR, is essential for the induction of SULT and PAPSS2 in vivo. A, the creation of PXR/CAR double-knockout mice. The absence of both PXR and CAR mRNA was confirmed by Northern blot analysis. An ethidium bromide staining of the agarose gel was shown to indicate an equal loading. B, The $PXR^{+/-}$ or PXR-null mice were subjected to a single intraperitoneal injection of solvent (lanes 1 and 2), PB (lanes 3 and 4), or TCPOBOP (lanes 5 and 6). Total liver RNAs were subject to Northern blot analysis. The membranes were first probed for SULT2A9 and PAPSS2 and subsequently stripped and reprobed for GAPDH as a loading control. The $PXR^{+/-}$ mice had the same SULT2A9 basal expression and PB- and TCPOBOP-inducibility as their wild type littermates (data not shown). C, regulation of SULT2A9 and PAPSS2 in PXR-null (lanes 1 and 2), CAR-null (lanes 3 and 4) and CAR/PXR double-knockout (lanes 5 and 6) mice. C, vehicle control; T, TCPOBOP.

Sulfation by SULTs is known to play a critical role in the metabolism of many drugs, including the most commonly used anti-inflammatory agent, acetaminophen (Tylenol), whose overdoses are among the leading causes for clinical acute liver failure. Zhang et al. (2002) recently identified CAR as a key regulator of acetaminophen metabolism and hepatotoxicity. CAR activators induced the expression of several acetaminophen-metabolizing enzymes, including the glutathione *S*-transferase Pi, a phase II enzyme that inactivates the toxic acetaminophen quinone metabolite (Zhang et al., 2002). Our results suggest that the induction of SULTs may also contribute to the CAR-mediated xenobiotic response in our body's handling of acetaminophen exposure.

It is known that inherited differences in the enzymatic activity of sulfotransferases are likely to influence cancer risk. For example, several studies have shown that estrogen receptor-positive breast cancer cells have very low estrogen sulfotransferase activity (Falany and Falany, 1996). In addition to sulfonating the growth-promoting steroid hormones, SULTs have been shown to catalyze the sulfation of a wide array of chemical carcinogens. Indeed, sulfation of the carcinogenic *p*-nitrophenol is markedly increased in VP-CAR mice (Fig. 3E). Thus, the creation of these transgenic mice not only demonstrates a role for CAR in SULT regulation but also provides a potential in vivo model to assess the molecular dynamics of carcinogenesis and the contribution of sulfation to this process. Although sulfation typically leads to detoxification, certain xenobiotics can become mutagenic once sulfonated (Glatt, 1997).

We used the unique VP-CAR transgenic system to identify and characterize the cytosolic SULTs as targets of CAR. Ligand-facilitated target gene identification using wild-type or gene knockout mice has been widely used (Maglich et al., 2002; Ueda et al., 2002). We consider the use of the VP-fusion receptor transgenes to have unique advantages over drug treatment. This is particularly important because we now know that treatments with receptor pan-agonists, such as bile acids, may affect multiple receptors depending upon the tissue context (Staudinger et al., 2001; Xie et al., 2001; Makishima et al., 2002). Moreover, several lines of evidence suggest that ligand treatment may have additional transcrip-

tional consequences independent of the presence of endogenous receptor. For example, Ueda et al. identified 168 differentially expressed tags in response to PB treatment. However, nearly half of these tags were similarly affected in the CAR knockout mice (Ueda et al., 2002). Bypassing the requirement of ligand treatment, the VP fusion of receptors provides a unique strategy not only to study the biological consequences of receptor activation but also to identify target genes (Rosenfeld et al., 2003). The utility and practicality of this strategy have been proven in our previous creation and characterization of the Alb-VP-hPXR (previously known as VPSXR) transgenic mice, in which the activated hPXR was expressed in the liver (Xie et al., 2000b). Even though the VP fusion receptor of CAR represents a unique tool to genetically dissect the gene regulation by CAR, we recognize that the level of CAR expression and/or activity in the VP-CAR mice may be substantially higher than the endogenous CAR activity in response to endogenous ligands in normal physiology. However, the limitation of this genetic model does not exclude the potential that pharmacological modulation of CAR activity may be applied to detoxify bile acids.

In addition to CAR, several other orphan receptors have also been implicated in the regulation of *SULT* gene expression. For example, we have recently reported the DHEA SULT as a direct transcriptional target of PXR in response to bile acids and many other PXR ligands (Sonoda et al., 2002). FXR, a prototypic bile acid receptor, was also shown to regulate DHEA SULT in cultured cells (Song et al., 2001). Thus, three distinct nuclear receptors, CAR, PXR, and FXR, may collaborate to regulate the sulfation cascade to detoxify xeno- and endotoxins. Interestingly, all three receptors use the same IR0 response elements found in *SULT2A* gene promoters. This represents another example of the sharing of binding sites by xenobiotic nuclear receptors, the underlying mechanism of the proposed "fail-safe pathways" in xenobiotic regulation (Xie et al., 2000a). Intriguingly, although CAR is both necessary and sufficient for SULT induction, loss of CAR and PXR individually or in combination does not suppress the basal expression of SULT (data not shown). It is possible that the sustained basal expression of SULT2A9 in CAR/PXR double-knockout mice is mediated, at least in part, by FXR.

Last but not least, the Tet-Off transgenic system has many attributes to facilitate the study of nuclear receptor functions in vivo. Because of their critical roles in development and normal physiology, embryonic and perinatal lethality is not unusual when genes encoding nuclear receptors or its genetic variants were disrupted via homologous recombination or overexpressed through transgenes. The concept of conditional expression was conceived to overcome the many potential circumstances of embryonic and perinatal lethality that accompany changes in the expression of many important genes (Xie et al., 1999). Tetracycline/doxycycline regulated systems seem to provide a solution. As to the study of xenobiotic receptors, not only can the application of inducible systems overcome potential lethality but it can also effectively establish the role of specific xenobiotic receptors in drug metabolism, drug-drug interactions, and drug toxicity. For example, the reversibility of VP-CAR expression and the resultant SULT induction and protection against xenotoxicants can be applied to study the effect of SULT activity on xenobiotic clearance in a developmental stage- and chemical exposure-specific manner.

Although CAR itself exhibits certain levels of constitutive activity, the VP-CAR is fundamentally different in that the VP-CAR seems to bypass the biological system by directly transactivating genes in the nucleus, whereas the wild-type CAR normally resides in the cytoplasm. Compared with PXR, although CAR is called a "xenobiotic receptor", it actually does not bind most of the ligands that activate it. Thus, CAR may function as a sensor for the class of xenobiotic compounds that act through a cell surface pathway to trigger CAR translocation from the cytosol to the nucleus. PXR directly binds bile acids but CAR does not. Therefore, we believe that the ability of CAR to control bile acid homeostasis is more than a simple extension of the PXR function. The development of inducible VP-CAR transgenic system provides a unique approach to further dissect the CAR-regulated mammalian xenobiotic response.

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HUMAN PXR VARIANTS AND THEIR DIFFERENTIAL EFFECTS ON THE REGULATION OF HUMAN UDP-GLUCURONOSYLTRANSFERASE GENE EXPRESSION

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ABSTRACT:

The pregnane X receptor (PXR) has three known major transcript variants resulting from alternative splicing. The less well characterized variants T2 and T3 are identical to the well described variant T1 except for a 39-amino acid N-terminal extension in T2 and an internal 37-amino acid deletion in T3. We have developed reverse transcription-polymerase chain reaction (RT-PCR) methods to detect and quantify each human PXR (hPXR) in human liver and intestinal tissues and HepG2 and Caco-2 cell lines. All three isoforms were expressed in hepatic cells, whereas only T1 transcripts were found in Caco-2 cells. In general, most normal human liver and intestinal mucosa contained all three hPXR variants, but considerable interindividual variation in expression levels was found. The effect of each hPXR variant on expression of UDP-glucuronosyltransferase (UGT) UGT1A and UGT2B family isoforms was investigated in transiently transfected HepG2 and Caco-2 cells. As a family, UGT1A transcripts were up-regulated by T1 and T2 but not T3. Isoform-specific RT-PCR revealed that UGT1A1, 1A3, and 1A4 were the major isoforms induced in both cell lines. The levels of several UGT1A isoforms were also examined in human liver samples from a number of donors with characterized PXR expression. The data suggest that individual variation in PXR expression may account for differential expression of some UGT isoforms between subjects.

The pregnane X receptor (PXR)[2], a ligand-regulated orphan nuclear receptor, has been identified as a species-specific xenobiotic receptor (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998). This receptor is activated by natural and synthetic pregnenolone derivatives and by a large number of structurally diverse compounds such as rifampicin (RIF), hyperforin (a constituent of St. John's wort), and bile acids (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer and Willson, 2002). Molecular studies have revealed that PXR is a key transcription factor responsible for CYP3A4 and CYP3A7 induction (Bertilsson et al., 1998; Blumberg et al., 1998; Pascussi et al., 1999; Xie et al., 2000; Staudinger et al., 2001; Kliewer and Willson, 2002) as well as some important efflux transporters, including multidrug resistant proteins 1 and 2 (Synold et al., 2001; Kast et al., 2002).

Several variants of human PXR (hPXR) have been previously identified. Blumberg et al. (1998), Bertilsson et al. (1998), and Lehmann et al. (1998) simultaneously isolated a cDNA variously termed SXR (steroid and xenobiotic receptor), hPAR-1, and hPXR (now known as T1). Northern blot analysis demonstrated that this mRNA is expressed at high levels in liver and moderate levels in intestine. Translation yields a protein of 434 amino acids, with a predicted molecular weight of 50,000. Concurrently, Bertilsson et al. (1998) isolated two cDNAs, T1 and hPAR-2 (T2). T2 cDNA differs from T1 at the 5′ end, resulting in an open reading frame 39 amino acids longer. A third hPXR variant mRNA (T3), containing an in-frame deletion of 111 nucleotides (823-933 relative to T1) was first described by Dotzlaw et al. (1999). T3, along with T1, was found to be expressed in normal and neoplastic breast tissue. T3 is similar to mouse PXR.2, which contains an in-frame 123-nucleotide deletion in a similar region of the ligand-binding

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[2] Abbreviations used are: PXR, pregname X receptor; RIF, rifamppicin; hPXR, human PXR; NR, nuclear receptor; UGT, UDP-glucuronsytransferase; DMEM, Dulbecco's modified Eagle's medium; RT-PCR, reverse transcription-polymerase chain reaction; dNTP, deoxynucleoside-5′-triphosphate; UTR, untranslated region; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; P450, cytochrome P450.

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domain (Dotzlaw et al., 1999). Mouse PXR.2, compared with mouse PXR.1 (analogous to T1), showed a reduced response to agents that could activate the wild-type receptor in transient transfection analyses. It is possible that human PXR variants may display a similar profile or be unable to bind ligand. Hustert et al. (2001) showed that T3 expressed in the human colon adenocarcinoma cell line, LS174T, did not direct transcription from the CYP3A4 promoter.

There is currently little information relating the function of nuclear receptors (NRs) to transcriptional activation of UGTs, and none regarding the roles of individual PXR variants. There are marked interindividual differences in the UGT content of the liver, intestine, and other organs, which are postulated to be the result of differential transcription. Depending on the substrate, variations in UGT activity of 6- to 15-fold in liver microsomes and 10- to 100-fold in intestinal microsomes have been found. Similar variations in UGT protein content have been demonstrated by Western blot in liver and intestinal microsomes (Burchell and Coughtrie, 1997; Little et al., 1999, 2002; Strassburg et al., 1999; Court et al., 2001; Antonio et al., 2003). A similar degree of variability in hepatic UGT mRNA levels has also been reported (Congiu et al., 2002). In addition, UGTs are distributed in a tissue-specific manner throughout the body. UGT1A1, 1A3, 1A4, 1A6, 1A9, and all the UGT2B isoforms have been shown to be expressed in the liver (Strassburg et al., 2000; Turgeon et al., 2001). In comparison, human intestine has been shown to express UGT1A1, 1A6, 1A8, 1A10 (Strassburg et al., 2000; Tukey and Strassburg, 2001), and UGT2B7 (Radominska-Pandya et al., 1998; Czernik et al., 2000). The intestine harbors many phase I and phase II enzymes and thus plays an essential role in the metabolism and detoxification of xenobiotics (see Tukey and Strassburg, 2001, for a review). Therefore, one factor influencing the tissue-specific UGT expression pattern may be the expression levels of NR proteins, in particular PXR, and the availability of their ligands.

In the present work, we have carried out the first systematic studies of hPXR variant mRNA levels in human liver and intestine as well as human hepatic and intestinal cell lines. We also describe for the first time the effect of the individual hPXR variants on UGT expression in HepG2 and Caco-2 cells. Two UGT isoforms, 1A3 and 1A4, were identified as new hPXR target genes. In contrast, the UGT2B isoforms were not responsive to PXR under the experimental conditions used. Finally, we investigated the association among the expression levels of T1, T2, and T3 hPXR mRNAs and UGT1A in human liver from several donors. We postulate that the varying levels of the natural PXR protein variants, combined with their differential transcription potentials, may have an important impact on both tissue-specific and interindividual target gene expression profiles. Furthermore, with the identification of two new target genes and elucidation of the PXR variants that are responsible for UGT regulation, our results give new insight into the role that PXR may play in UGT-related drug metabolism and clinical drug-drug interactions.

Materials and Methods

Cell Culture and Transient Transfections. The human hepatocellular carcinoma cell line, HepG2 (ATCC HB-8065) and human adenocarcinoma cells line, Caco-2 (ATCC HTB-37), were obtained from the American Type Culture Collection (Manassas, VA). Both cell lines were maintained at 37°C, 5% CO_2 in high glucose Dulbecco's modified Eagle's medium (DMEM) with Earle's salts and L-glutamine (Invitrogen, Carlsbad, CA), supplemented with 1% nonessential amino acids, 1 mM sodium pyruvate, and 10% fetal bovine serum (Invitrogen or Trace Biosciences, Sydney, Australia). The culture medium was changed twice weekly during maintenance. Untransfected cells used for RNA isolation were harvested when they neared confluence. Caco-2 cells were received at passage number 18; the HepG2 cells were received at passage number 77. The Caco-2 cells were stable over the period of the experiments.

For transient transfection, HepG2 or Caco-2 cells were seeded in six-well plates at 1×10^6 and 2.5×10^5 cells per well, respectively. Transfections were performed in triplicate using 5 μg of expression plasmid or empty parent vector and 10 μl of LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. Six to seven hours after transfection, the cells were washed in DMEM and replacement medium containing 10 μM RIF (Sigma-Aldrich, St. Louis, MO) or vehicle (0.1% dimethyl sulfoxide) was added. At 24 h post-transfection, the cells were treated again with RIF or solvent in fresh DMEM. After a total of 40 h, all transfections were washed in phosphate-buffered saline and harvested for RNA extraction. Using the protocols of the manufacturer, the transfection efficiencies were on average about 70%.

Human tissue samples. The human intestinal tissues used in the present studies were obtained from organ donors (details are summarized in the legends to the appropriate figures) by transplant surgeons at University Hospital, Little Rock, AR, according to a protocol approved by the Human Research Advisory Committee of the University of Arkansas for Medical Sciences. The intestines were received from the surgeons in saline on ice. Working at 4°C, the small intestine was divided into four segments of 80 to 100 cm in length, and the colon was treated as a single segment. Each segment of intestine was opened, the contents were removed, and the tissue was rinsed in cold 0.9% NaCl. Mucosa was removed from each segment by scraping with a glass slide, and RNA was prepared as described below.

Two of the human livers used in these studies were also obtained from the transplantation program at the University of Arkansas for Medical Sciences. The remaining liver samples were a generous gift from Dr. Mary Relling at St. Jude Children's Research Hospital (Memphis, TN). Again, donor information is given in the legends to the appropriate figures. mRNA extraction is described below.

RNA Isolation. Total RNA was isolated from 50 mg of frozen tissue or untransfected cell culture using a phenol and guanidine isothiocyanate RNA extraction method following the instructions of the suppliers (Trizol; Invitrogen). Total RNA from transfection experiments was harvested using the RNeasy Mini Kit (QIAGEN, Valencia, CA), per the instruction manual. To avoid any contamination of the RNA by genomic DNA, RNA samples were treated with RNase-free DNase (RQ1; Promega, Madison, WI).

Plasmids and Cloning of hPXR Variants. Plasmids for expression of hPXR variants T1, T2, and T3 were constructed by insertion of PCR-amplified cDNAs (described below) into the XbaI and HindIII sites of pCMV5. Each cDNA was confirmed by sequencing and was subsequently transferred to the pCMX vector that was used to create the T1 expression vector. The pCMV5- and pCMX-based vectors showed no discernible difference in expression of the PXR variants. The sources of the pCMX and pCMV5 vectors have been described elsewhere (Andersson et al., 1989; Xie et al., 2000).

PXR T1 and T2 cDNAs were amplified from HepG2 cDNA using the common reverse primer PXRrc (agccatctagatcagctacctgtgatgccgaa) and specific primers for the 5′ ends, PXRT1 and 3 (agccataagcttatggaggtgagaccaaaga) and PXRT2 (agccataagcttatgacagtcaccaggactca). The CTG initiation codon of T1, and consequently T3, was replaced by the more conventional ATG through primer mismatch. Restriction sites are highlighted in bold. All PCRs for cloning were done with 1.25 units (U) of *Pfu Turbo* DNA polymerase (Stratagene, La Jolla, CA) in 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 1% Triton X-100, and 0.1 mg/ml bovine serum albumin with 0.5 mM dNTPs and 50 ng of each primer in a total volume of 20 μl. After an initial 4 min at 95°C, 40 cycles of 95°C, 45 s; 60°C, 45 s; and 72°C, 4 min were performed. Each PCR was finished with 5 min at 72°C.

T3 cDNA was synthesized from the newly cloned T1 cDNA by overlap PCR. T3 has a sequence identical to T1 except for an internal 111 base pairs. Primers spanning this junction, aagaatttccgggtctctctgcagctgcgg and ctgcagagagacccggaaattcttgaaatggga, were used in combination with PXRT1, -3, and PXRrc to amplify the remainder of T1 and fuse the two sections together.

Semiquantitative RT-PCR for hPXR Variants. cDNA was synthesized by mixing 1 μg of total RNA from each sample with 100 pmol random hexamers in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 100 U Moloney murine leukemia virus-reverse transcriptase, 20 U RNase inhibitor, and 1 mM each dNTP in a total volume of 20 μl (Promega).

TABLE 1

PCR primers and conditions for semiquantitative analysis of PXR variants and PXR-induced transcription

Nucleotide sequences are reported for primer pairs used in RT-PCR analysis of PXR transfection experiments in HepG2 and Caco-2 cells. Two common primers are used for the UGT2B isoform PCR: cR1 has two mismatches to UGT2B4 and one mismatch each to UGT2B7, UGT2B11, and UGT2B15. cR2 has one mismatch to UGT2B10 and three mismatches to UGT2B28. The nucleotide sequences for UGT1A4F, UGT1A6F and R, UGT1A10R, and all UGT2B primers except UGT2B28F were obtained from Congiu et al. (2002). The sense primer for UGT1A10 was designed by Strassburg et al (1997). PXR variant primers were designed by J.-M. Heydel.

Target Transcript	Primer Set	Annealing Temp	Amplicon Size	No. of Cycles on HepG2 cDNA	No. of Cycles on Caco-2 cDNA
		°C	*bp*		
T1.T2.T3	FC: 5′ agaaggagatgatcatgtccga 3′ RC: 5′ gtttgtagttccagacactgcc 3′	60	359 248	32	34
T1	F1: 5′ caagccaagtgttcacagtgag 3′ R1: 5′ caaagagcacagatcttccg 3′	60	818	35	35
T2	F2: 5′ gcagcatgacagtcacc 3′ R2: 5′ ctccttcttcatgccgctct 3′	62	460	35	nd
T3	F3: 5′ cactgcctttacttcagtggg 3′ R3: 5′ cagctgcagagagacccg 3′	60	764	35	nd
PXR	F: tgtcatgacatgtgaaggatg R: ttgaaatgggagaaggtagtg	58	327	25	25
CYP3A7	F: agttgctatgagacttgagag R: aatctacttccccagcactga	50	637	30	32
UGT1A	F: tgaaagcatatgcaatggcgt R: tcaatgggtcttggatttgtg	50	466	30	27
UGT1A1	F: atgctgtggagtcccagggc R: ccattgatcccaaagagaaaacc	50	932	30	30
UGT1A3	F: atggcaatgttgaacaatatg R: ggtctgaattggttgttagtaatc	58	247	35	35
UGT1A4	F: acgctgggctacactcaagg R: gacaggtacttagccagcacc	66	200	40	35
UGT1A6	F: cttttcacagacccagccttac R: tatccacatctctcttgaggacag	58	289	42	25
UGT1A7	F: tggctcgtgcagggtggactg R: ttcgcaatggtgccgtccagc	63	310	nd	35
UGT1A8	F: ctgctgacctgtggctttgct R: ccattgagcatcggcgaaat	63	248	nd	35
UGT1A9	F: gaggaacatttattatgccaccg R: ccattgatcccaaagagaaaacc	50	281	34	32
UGT1A10	F: cctctttcctatgtccccaatga R: gcaacaaccaaattgatgtgtg	63	205	nd	35
UGT2B	F: aagttctaggaagacccactac R: caccacaacaccattttctcca	58	205	30	na
UGT2B4	F: tctactcttaaatttgaagtttatcctgt cR1: tcagcccagcagctcaccacaggg	58	278	30	na
UGT2B7	F: agttggagaatttcatcatgcaacaga cR1: tcagcccagcagctcaccacaggg	58	232	26	30
UGT2B10	F: tgacatcgtttttgcagatgctta cR2: caggtacataggaaggagggaa	58	152	28	na
UGT2B11	F: cttccattctttttgatcccaatgatg cR1: tcagcccagcagctcaccacaggg	58	307	30	na
UGT2B15	F: gtgttgggaatattatgactacagtaac cR1: tcagcccagcagctcaccacaggg	58	141	32	na
UGT2B17	F: gtgttgggaatattctgactataatata cR2: caggtacataggaaggagggaa	58	242	41	na
UGT2B28	F: atcccaatgacgcattcactcttaaactc cR2: caggtacataggaaggagggaa	58	340	nd	na
β-actin1	F: ctggcggcaccaccatgtaccct R: ggaggggccggactcgtcatact	50	205	18	18
β-actin2	F: cgtaccactggcatcgtgat R: gtgttggcgtacaggtcttt	58	452	18	18
GAPDH	F: 5′-acccactcctccacctttg-3′ R: 5′-ctcttgtgctcttgctggg-3′	64	178	25	25

F, forward primer; R, reverse primer; bp, base pair; nd, not detected; na, not attempted.

The samples were incubated at 37°C for 60 min and then diluted to 100 μl with sterile diethylpyrocarbonate-treated H_2O. The reverse transcriptase was inactivated by heating at 95°C for 5 min.

The primers used to detect hPXR wild-type T1 (NM_003889), T2 (NM_022002), and T3 (NM_033013) variants are described in Table 1. In addition to a common primer set designed to amplify all three hPXR transcripts, specific primers to detect them individually were made, based on differences between the splice variants. To distinguish T2 from T1 and T3, the forward primer F2 was designed within the 5′ sequence unique to this transcript (Fig. 1). In contrast, T1 and T3 share all of the sequence except the deleted region in T3; thus, the amplification specificity between these variants was facilitated by the design of the reverse primers. To exclusively amplify T1, the F1 primer was positioned in the 5′ untranslated region (UTR) absent in T2, and the reverse primer was designed within the 111-base pair sequence deleted in T3. To detect T3 only, another primer in the 5′ UTR of F3 was used, in combination with an oligonucleotide which binds over the boundary of the sequence missing in T3 as shown in Fig. 1. GAPDH amplification was used as an internal control (see Table 1).

The semiquantitative hPXR PCR reactions were performed as follows: a 10-μl cDNA aliquot was added to a reaction mixture containing 10 mM

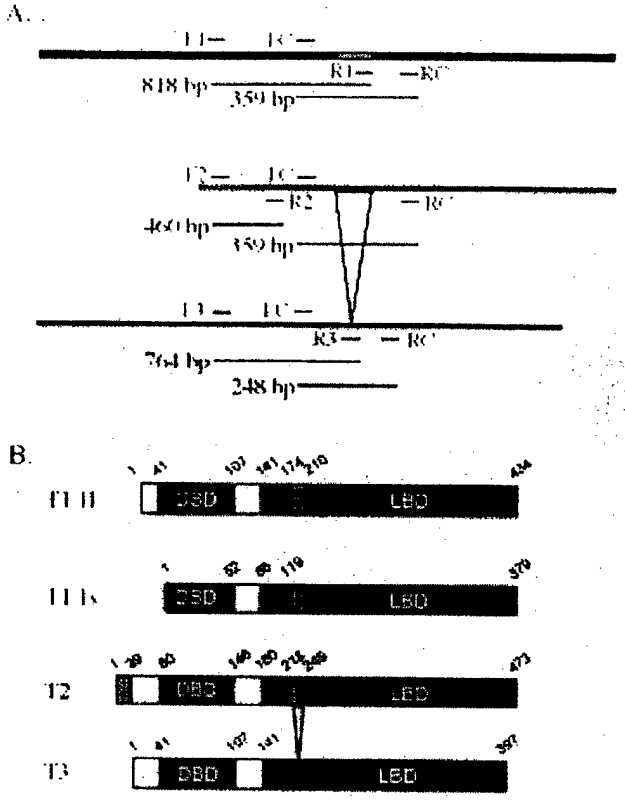

FIG. 1. *Structure of human PXR transcripts, design of transcript specific PCR primers, and natural protein variants of human PXR.*

A, transcripts were amplified specifically by RT-PCR using transcript-specific primer pairs. T1 originates from exon 1A and corresponds to the cDNA published as PXR wild type. T2, which has a unique 5′ UTR and start codon, begins in exon 1B and is the cDNA published as PAR2. T3 is similar to T1 but has an in-frame deletion of 111 base pairs at the 5′ end of exon 5. B, T2 is similar to T1 but contains an additional 39 amino acids on its N terminus. T3 is similar to T1 but has an internal 37-amino acid deletion. F is the forward primer and R is the reverse. F1 is the T1 forward primer and R1 is the T1 reverse primer, etc. RC is the reverse control primer. Also, DBD is the DNA-binding domain, and LBD is the ligand-binding domain.

Tris-HCl buffer (pH 8), 20 mM KCl, 0.1% Triton X-100; 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 50 pmol of each primer, and 2 U of *Taq*DNA polymerase (Promega), in a total volume of 50 μl. The mixture was subjected to 34 cycles consisting of a 45-s denaturing step at 94°C, a 45-s annealing step at 59°C, and a 45-s elongation step at 72°C in a thermal cycler (MJ Research, Reno, NV). Amplification of the ubiquitously expressed GAPDH cDNA was performed under the same conditions in separate experiments. Amplification products were resolved by agarose gel (2%) electrophoresis and detected by ethidium bromide. The bands were visualized under UV light and photographed with a computer-assisted camera. Quantification of each band was performed by densitometric analysis using NIH Image software (National Institutes of Health, Bethesda, MD). The identities of all PCR products were confirmed by sequencing.

Semiquantitative RT-PCR for CYP3A7 and UGT Detection. cDNA from transfection experiments was made from 1 μg (HepG2) or 0.6 μg (Caco-2) RNA, using the Invitrogen Superscript system. First-strand synthesis was performed using the oligo(dT) primer method according to the supplier's instructions. All completed cDNA reactions were diluted in diethylpyrocarbonate-treated water to the equivalent of 10 μg/ml of the original RNA.

To confirm successful transfection of the PXR expression vectors and the ability of each individual construct to overexpress a PXR variant, PCR with a common primer set (Table 1) was used. All primers in Table 1 were obtained from Sigma-Genosys (Castle Hill, Australia) or Integrated DNA Technology (Coralville, IA). PCR for experiments with cultured cells were performed with

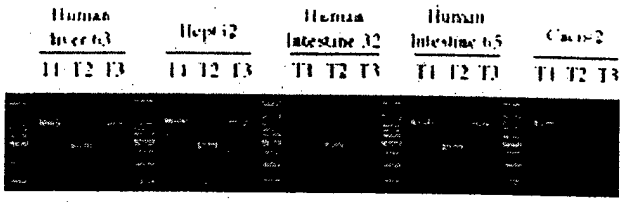

FIG. 2. *RT-PCR analysis of mRNA expression of hPXR variants in human tissues and cell cultures.*

Total mRNA from human liver, human intestine, and cell cultures was analyzed by RT-PCR. Primers specific for the hPXR variants T1, T2, and T3 were used to generate the amplicons. A DNA standard ladder is positioned in the first lane and between each tissue sample group.

0.5 U of *Taq* polymerase (Amersham Biosciences Inc., Piscataway, NJ) on 4 to 10 μl of cDNA under the following conditions: 95°C, 5 min, followed by 25 to 42 cycles of 30 s at 95°C; 30 s at an appropriate annealing temperature; 1 min at 72°C; and a final 5 min at 72°C. Table 1 details the annealing temperature and cycle number required for each template. Primer pairs were designed to specifically amplify across exon boundaries in mRNA from β-actin, CYP3A7, UGT1A1, UGT1A9, UGT2B7, and the UGT1A family as a whole (Table 1). For CYP3A7, UGT1A, and UGT2B7, PCR for each reaction was paused at 72°C with 18 cycles remaining and 50 ng of both β-actin1 primers were added as an internal reference. For the remainder of the semiquantitative reactions, β-actin2 control PCR was performed as a separate reaction. The specificity of all primer pairs was confirmed through sequencing or restriction analysis of the PCR products. Semiquantitative analysis of each PCR product was as described above, using Molecular Analyst software (Bio-Rad, Hercules, CA).

Results

Identification of hPXR mRNA Variants in Human Tissues and Cell Lines. Estimation of mRNA levels of hPXR transcripts in human tissues and two human cell lines was carried out by semiquantitative RT-PCR. One human liver sample, intestinal mucosa from the jejunum of two donors, and HepG2 and Caco-2 cells were examined (Fig. 2). The expected PCR products for hPXR T1, T2, and T3 variants were detected in both the representative normal human liver and the human hepatoma cell line, HepG2 (Fig. 2). The expression pattern in these samples was almost identical. In contrast, the pattern of variant expression in the jejunal segments from the two selected donors was strikingly different: in one donor (human intestine 32), only the T2 variant was detected, whereas in the second donor (human intestine 65), the pattern of expression was similar to that of the liver (Fig. 2). Examination of Caco-2 cells showed that only T1 was present in this human intestinal cancer cell line, an obvious difference in comparison with human jejunal mucosa.

Induction of CYP3A7 and UGT Genes by PXR Variants in HepG2 Cells. Since it is well established that RIF, via PXR, induces P450s, we selected one isoform, CYP3A7, as a model for comparison with UGT induction studies. Cells transfected with control plasmid pCMV5 responded to the addition of RIF with a modest increase in CYP3A7 transcription (Fig. 3A). Cotransfection of PXR variant 1 in the presence of RIF enhanced this up-regulation approximately 4-fold relative to the original basal expression levels. Interestingly, hPXR T2 was as effective as T1 in mediating CYP3A7 induction, and T3 had no effect on transcription of any of the genes studied. This experiment was carried out to ensure that the HepG2 cells responded correctly to the inducer and no quantitation was attempted.

There was a very marked increase in UGT1A transcripts in HepG2 cells transfected with either T1 or T2 and subsequently treated with RIF. When expressed in the presence of 10 μM RIF, these two PXR variants were each responsible for increased transcription of the

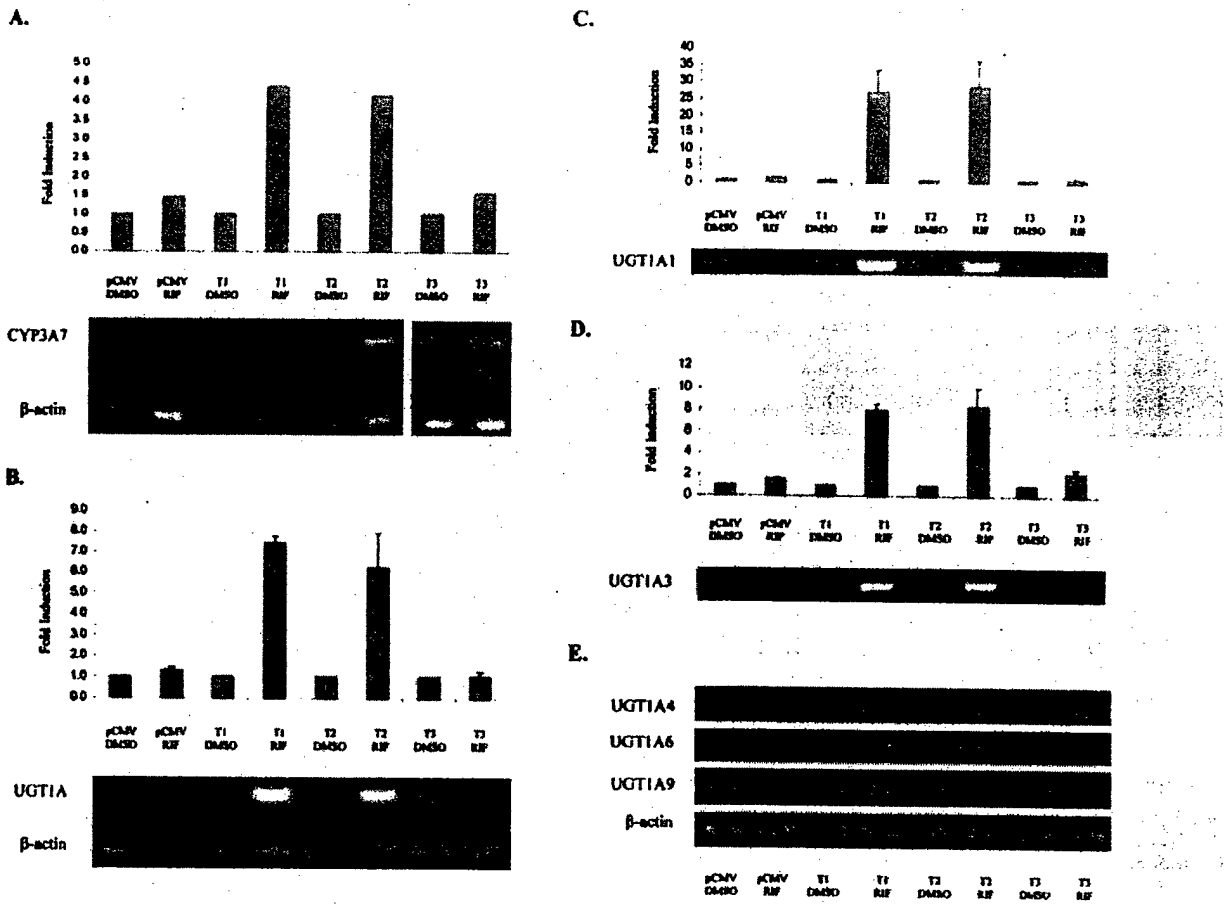

FIG. 3. *Induction of CYP3A7 and the UGT1A gene family members by hPXR in a hepatocellular carcinoma cell line.*

HepG2 cells were transfected with plasmids containing hPXR variants and treated with 10 μM RIF or solvent control. Total RNA harvested from cells exposed to each treatment was analyzed by RT-PCR for altered transcriptional regulation of CYP3A7 (A), UGT1A family (B), UGT1A1 (C), UGT1A3 (D), and UGT1A4, 1A6, and 1A9 (E). Results have been normalized by comparison with the expression of β-actin and are expressed as the mean (n = 3) ± S.D. The PCR shown in Fig. 3A is a control experiment carried out only once to ensure that the HepG2 cells responded correctly to the inducers as expected. The experiments in Fig. 3E were not quantitated since the bands were not clearly visible; therefore, only the raw data are presented.

UGT1A family by up to 7-fold (Fig. 3B). Interestingly, however, this augmented expression was found to be inconsistent among individual 1A family members, most likely due to the differential expression of UGT1A family members.

UGT1A1 appeared to contribute the most to the observed UGT1A family up-regulation in HepG2 cells by responding very considerably to the combined presence of either recombinant hPXR T1 or T2 and RIF. More than a 25-fold induction of UGT1A1 transcripts was observed under the conditions used (Fig. 3C). Similarly, UGT1A3 was strongly up-regulated by hPXR in response to RIF (Fig. 3D). Although baseline expression was low, UGT1A3 was observed to be induced to approximately 8-fold over controls. Again, T1 and T2 exhibited a similar potency for mediating up-regulation, whereas T3 showed no noteworthy activity (Fig. 3D).

Figure 3E illustrates that UGT1A6 and 1A4 were also responsive to PXR/RIF treatment, although to a much lesser extent than UGT1A1 and UGT1A3. In contrast, the level of UGT1A9 transcription remained unchanged by PXR transfection and RIF treatment. Production of UGT1A9 mRNA in HepG2 cells was not induced by cotransfection of constitutively active hPXR or human constitutive androstane receptor (data not shown). UGT1A7, 1A8, and 1A10 were also examined, but transcripts could not be detected in HepG2 cells. The data shown are representative of all replicates. However, the bands were too faint to quantify accurately, hence the raw data are shown.

In comparison with the UGT1A family, PXR-mediated induction of UGT2B transcripts was not detected. The ability of all three PXR variants to up-regulate UGT2B7 was examined, and no induction was observed. In addition, cotransfection of recombinant T1 had no effect on UGT2B4, 2B10, 2B11, 2B15, or 2B17 mRNA (data not shown). UGT2B28 transcripts could not be detected in HepG2 cells (data not shown), and the T2 and T3 PXR variants were not tested for regulatory interaction with any UGT2B gene other than UGT2B7. Therefore, it appears that hPXR, particularly variant T1, cannot direct transcription from the UGT2B promoter in HepG2 cells under the experimental conditions used. However, we cannot exclude the possibility that an assay of greater sensitivity may reveal subtle changes that currently remain undetected, but any such associations would be anticipated to be weak.

CYP3A7 and UGT Induction in Caco-2 Cells. Although the UGT1A expression profile in Caco-2 cells differed from that of HepG2 cells, the response of most individual genes to PXR/RIF treatment was similar, with some noteworthy exceptions (Fig. 4). UGT1A1 (Fig. 4A) was again strongly up-regulated by PXR variants T1 and T2, but to a lesser extent than in HepG2 cells. UGT1A3 and UGT1A4 also showed obvious responses to T1 and T2 but not T3

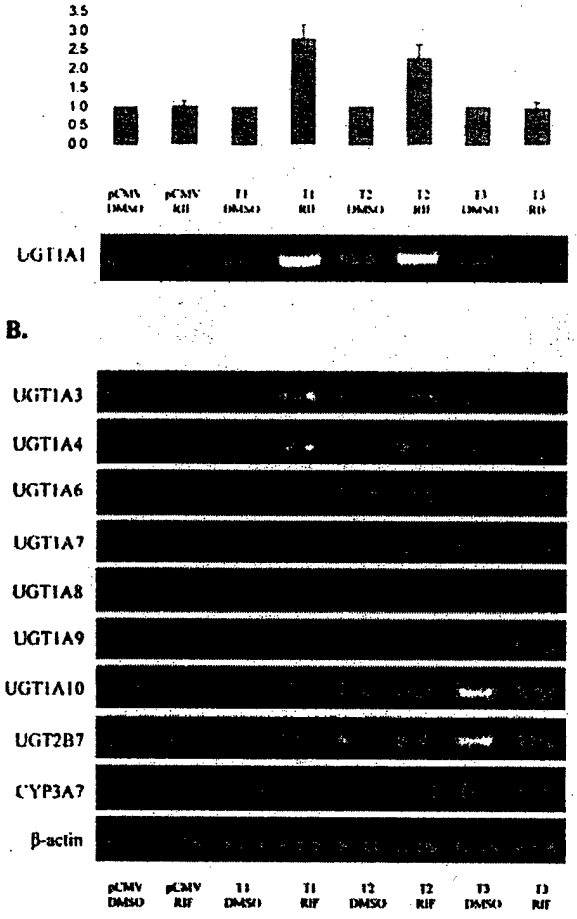

FIG. 4. *Transcriptional regulation of CYP3A7 and UGT genes by hPXR in a colorectal adenocarcinoma cell line.*

Caco-2 cells were transfected with expression constructs for hPXR variants T1, T2, or T3 and exposed to dimethyl sulfoxide or RIF as described in the text. After treatment, regulation of target genes was analyzed by RT-PCR using specific primers for the coding regions of UGT1A1 (A) and UGT1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, and CYP3A7 (B). Up-regulation of UGT1A1 transcripts is presented as the mean (n = 3) ± S.D.

(Fig. 4B). These modest but clear up-regulations were consistent between replicates of the experiment shown in Fig. 4 and were confirmed in additional independent transfections. UGT1A9 and UGT2B7 expression again remained unchanged regardless of treatment. Unlike HepG2 cells, UGT1A7, 1A8, and 1A10 mRNAs were detectable in Caco-2 cells. Like the closely related UGT1A9 gene, UGT1A7, 1A8, and 1A10 were not detectably increased by PXR and RIF. This experiment was carried out several times, and the results were not consistent among replicates. On average, there was no effect.

The only UGT1A isoform expressed in both HepG2 and Caco-2 cells, but found to differ in response to PXR/RIF treatment, was UGT1A6. UGT1A6 transcripts are present in Caco-2 cells at substantially higher levels than in HepG2 cells, yet the response seen in the latter to T1 and T2 (Fig. 3E) was not observed in Caco-2 culture (Fig. 4B). The other major difference observed between the two cell lines transfected with hPXR and subsequently exposed to RIF was the lack of CYP3A7 response in Caco-2 cells. CYP3A7 transcripts have been shown previously to be present at low levels in Caco-2 cells

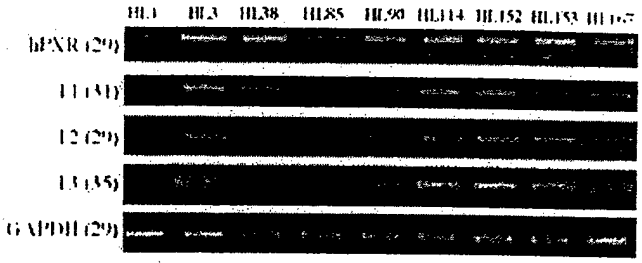

FIG. 5. *Individual variation of hPXR transcript expression in human liver samples.*

General and variant specific expression of hPXR in total RNA purified from nine human liver samples were analyzed by RT-PCR. To optimize transcript detection, each RT-PCR profile was obtained from reactions with differing numbers of cycles (in parentheses). H1 and H2 were, respectively, a 66-year-old female and a 49-year-old male, both of whom died of strokes. The remaining liver samples were from normal females ranging in age from 23 to 64. No further information was available for these donors. The PCR shown is representative of three to seven estimations.

(Schmiedlin-Ren et al., 1997) and are known to be responsive to rifampicin in other cell lines (this study; Pascussi et al., 1999), yet CYP3A7 did not respond appreciably to the addition of RIF, with or without PXR cotransfection. Therefore, it is evident that hPXR variants display cell line- and, most likely, tissue-specific activity. It has also been demonstrated, in all our studies, that when PXR T1 was active, T2, but not T3, also showed activity. Thus, the behavior among the individual variants relative to one another was consistent among all genes and the two host cells tested.

Variability of hPXR mRNA Expression Levels in Human Liver and Intestine. As variability in expression of hPXR T1, T2, and T3 variants could potentially influence the transcriptional regulation of target genes in human liver, we investigated mRNA expression in nine human livers derived from generally healthy donors. The mRNA levels of hPXR variants were measured by RT-PCR and normalized to the GAPDH mRNA level in each sample. The results obtained are shown in Fig. 5. RT-PCR with common primers, as well as those for the individual transcripts, showed great individual variations among donors. Overall, livers with high expression of one variant also had high expression of the other two variants. Interestingly, the UGT1A isoform whose expression correlates most with the expression of PXR was UGT1A9. However, we have not identified this enzyme as being up-regulated by any of the hPXR variants in HepG2 and Caco-2 cells. This phenomenon could be explained by the possibility that these cells do not have all the cofactors necessary for UGT1A9 transactivation by PXR.

We also investigated mRNA expression in six human intestinal (jejunum) segments from healthy donors. As noted previously, the mRNA levels of hPXR variants were measured by RT-PCR and normalized to the GAPDH mRNA level in each sample. As with the liver samples, the intestinal mucosa showed great individual variations between donors. mRNA expression for the individual donors was considerably different in terms of amount and distribution pattern (Fig. 6).

Finally, the expression of hPXR and UGT1A isoforms was compared, and the results are shown in Fig. 7. There was noticeable interindividual variation observed in the expression of UGT1A isoforms in different donors. In general, donors with a low level of one UGT isoform also had low levels of the other isoforms, and the reverse was also true. Although quantitative evaluation was not possible for this experiment, the level of expression of some UGT1A isoforms appeared to mimic hPXR transcript expression.

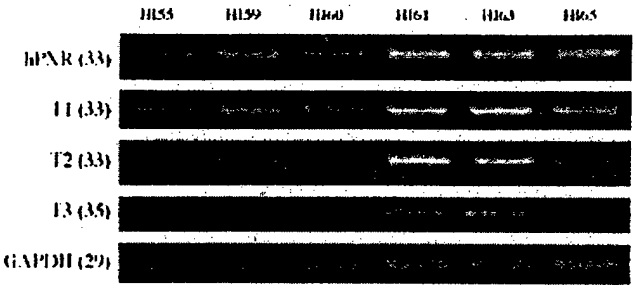

FIG. 6. *Individual variation of hPXR transcript expression in human intestine.*

General and variant specific expression of hPXR in total RNA purified from six human intestinal (jejunum) samples were analyzed by RT-PCR. To optimize transcript detection, each RT-PCR profile was obtained from reactions with differing numbers of cycles (in parentheses). Donors were as follows: H55, 49-year-old male, died of a stroke; H59, 19-year-old male; H60, 21-year-old male; H61, 47-year-old male; H63, 39-year-old male; H65, 22-year-old male; all of whom died in motor vehicle accidents.

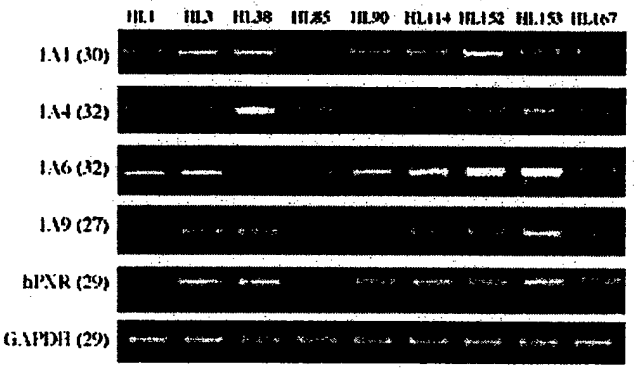

FIG. 7. *Expression of several UGT1A family members mimics hPXR transcript expression.*

RNA isolated from human liver samples was analyzed by RT-PCR to examine levels of expression for several UGT1A family members. For comparison, hPXR expression was also analyzed from the same liver samples. To optimize transcript detection, each RT-PCR profile was obtained from reactions with differing numbers of cycles (in parentheses). The source of the liver samples is described in the legend to Fig. 5.

Discussion

The results presented here support the recent report that PXR is capable of regulating the expression of UGT1A family isoforms and are in agreement with our previous results (Xie et al., 2003). Here, we show that two additional UGTs, UGT1A3 and UGT1A4, are also up-regulated by PXR variants. This finding suggests an important role for this xenobiotic receptor in the ligand-dependent activation of human UGTs. Our data also demonstrate that there are different mechanisms involved in the regulation of hepatic and intestinal UGTs. This would be consistent with the presence of distinct liver- and intestine-specific UGT isoforms.

Several laboratories have demonstrated the presence of natural variants of hPXR (Bertilsson et al., 1998; Kast et al., 2002), some of which possess altered transactivation activity toward P450 genes (Lehmann et al., 1998; Hustert et al., 2001). However, no systematic studies have been performed yet on the tissue distribution and function of these variants or their role in regulation of human UGTs. In these studies, we have analyzed the available PXR amino acid sequences and designed primers for the identification of three human PXR mRNAs, corresponding to the wild-type hPXR (T1) and variants with 39 extra N-terminal amino acids (T2) or an internal 37-amino acid deletion (T3). We have also cloned all three PXR variants and investigated their effect on regulation of UGTs from both the UGT1A and 2B families, as well as CYP3A7. Moreover, we have carried out the first systematic studies of the expression of these variants in human liver and intestine and in the human derived cell lines, HepG2 and Caco-2. It has been suggested that hPXRs are expressed in a restricted number of tissues, with the highest expression observed in the liver, followed by small intestine and colon (Bertilsson et al., 1998). Generally, hPXR variant expression has been analyzed in human tissue by Northern blot, and hPXR mRNAs of different sizes have been detected in both liver and intestine; however, in the latter tissue, hPXR expression was limited to the cells of the intestinal mucosal layer (Bertilsson et al., 1998). In addition, one earlier study has described the expression of PXR T1 and T3 by RT-PCR in normal and cancerous breast tissue (Dotzlaw et al., 1999).

Our data demonstrate that variants of human PXR are expressed in human liver, HepG2 cells, intestinal mucosa, and CaCo-2 cells; however, the pattern of distribution varies considerably between hepatic and intestinal tissue. All the variants were expressed in human liver and HepG2 cells. However, distribution in human intestine and Caco-2 cells was very different. In jejunum from H32, only T2 was expressed while, in the same tissue from H65, the pattern was identical to that of liver. Variants T2 and T3 were missing in Caco-2 cells. This indicates important tissue-specific distribution of hPXR variants and reflects the differential regulatory function of these variants.

We also investigated the effect of hPXR variant expression on the regulation of UGTs and CYP3A7 in HepG2 and Caco-2 cells. Interestingly, the same genes were up-regulated in both cell lines. However, the rate of up-regulation and response to RIF was somewhat different. In HepG2 cells, UGT1A1 responded quite strongly to the combined presence of T1/T2 and RIF. These data are consistent with a recent report by Xie et al. (2003), which showed that UGT1A1 is up-regulated in mice bearing constitutively activated hPXR. The same investigators also showed that hPXR and human constitutive androstane receptor can induce UGT1A1 promoter reporter gene expression in HepG2 cells. The UGT1A1 response to PXR T1 and T2 in the presence of RIF mirrors that of the UGT1A family as a whole. This is because, first, UGT1A1 is strongly induced by RIF and, second, UGT1A1 appears to be the major UGT isoform expressed in these cells. Thus, UGT1A1 up-regulation may be the major contributor to the up-regulation of the UGT1A family as a whole. However, UGT1A3 expression was also extensively increased, and UGT1A4 and 1A6 expression may contribute to the up-regulation as well.

Interesting data were also obtained on the regulatory effect of PXR variants in Caco-2 cells. When transfected with either T1 or T2 and treated with RIF, there was a marked increase in UGT1A1 transcription in these intestine-derived cells. Also, UGT1A3 transcription was increased by PXR/RIF. Like UGT1A3, UGT1A4 transcripts in CaCo-2 cells were clearly increased in the cells in response to T1 and RIF. Interestingly, UGT1A6, which, in our hands, is the most highly expressed UGT1A isoform in Caco-2 cells, was found not to be regulated by T1, with or without RIF. Thus, the high levels of endogenous UGT1A6, in conjunction with its unresponsiveness to PXR regulation, are likely to be responsible for the smaller response of the UGT1A family seen in Caco-2 cells relative to that of HepG2 cells. UGT1A8 and 1A10, whose expression is limited to the intestine, did not respond to PXR/RIF in Caco-2 cells. Moreover, none of the PXR variants in combination with RIF was able to influence UGT1A9 expression in Caco-2 cells.

In summary, the cell culture studies demonstrated that PXR T1 and T2 can up-regulate the UGT1A family of isoforms, in particular UGT1A1, UGT1A3, and UGT1A4. PXR T3 does not mediate up-regulation of any of the genes studied. None of the UGT2B isoforms

responded to any of the hPXR variants. Although HepG2 and Caco-2 cells have relatively low expression of endogenous UGT genes, they seem to be excellent models for studying hPXR-mediated regulation. This is especially true for UGT1A3 and UGT1A4, as both genes are expressed at low levels in these cell lines; however, both isoforms can be effectively up-regulated by PXR. This work has also shown, for the first time, that PXR regulates not only UGT1A1 but also UGT1A3 and UGT1A4. The different responses of UGT1A isoforms, varying from strong to undetectable, suggests differing regulation of these is under the control of its own unique promoter (Ritter et al., 1992). From these observations, it can be concluded that, like P450 enzymes and drug transporters, UGTs are also strongly regulated by xenobiotic NRs. In addition, the present studies have defined an important role of PXR in tissue specific regulation of UGTs.

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Orphan nuclear receptor-mediated xenobiotic regulation in drug metabolism

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[abstract]

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The past 50 years have witnessed a monumental advance in our understanding of pharmacodynamics (what drugs do to the body) and pharmacokinetics (what the body does to drugs). In the realm of pharmacokinetics, dozens of enzymes responsible for drug biotransformation and transporters responsible for the absorption, distribution and excretion of drugs have been identified.

Drug biotransformation (metabolism) is traditionally classified as either phase I or phase II. Phase I metabolism (functionalization) includes oxidation, reduction, hydrolysis and hydration. Enzymes catalyzing these reactions are found in virtually all tissues but especially in the hepato-intestinal axis. Quantitatively, however, the liver is generally considered to be the most important organ involved in drug metabolism. Located in the endoplasmic reticulum of hepatocytes is a family of heme proteins known as cytochrome P450 (P450 or CYP). CYP is the central constituent of the so-called microsomal mixed-function oxidase system. The components of this system catalyze the splitting of molecular oxygen with one atom being inserted into the drug molecule and the other undergoing reduction to water. The human genome encodes 57 CYP proteins so there is a substantial genetic component to the process of drug metabolism [1]. Moreover, the activity of CYP enzymes can be induced or inhibited by a variety of environmental chemicals and drugs, adding to the variability in metabolism of different individuals. The products of phase I metabolism are generally more polar and more readily excreted than the parent compounds and are often substrates for phase II enzymes. Phase II metabolism involves conjugation with endogenous hydrophilic compounds to further increase polarity and water solubility, and therefore drug excretion. Phase II metabolism is also subject to genetic and environmental variability. Although hepatic drug metabolism has been traditionally equated with 'detoxification', it is now known that in some cases highly reactive metabolites can be formed that react with crucial cellular macromolecules leading to various forms of toxicity.

Although metabolizing enzymes are important in the process of drug disposition, equally important are a group of transporter proteins that are expressed in various tissues, such as the intestine, brain, liver and kidney, which modulate the absorption, distribution and excretion of many drugs. These transporters are classified as either primary, secondary or tertiary. Primary transporters are driven by energy from ATP hydrolysis, whereas secondary and tertiary active transporters are driven by an exchange of intracellular ions. Like the

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drug metabolizing enzymes, each transporter gene family is composed of a multiplicity of members. These proteins control, among other things, absorption of many drugs from the gastrointestinal tract, exclusion of drugs from the brain (a component of the blood–brain barrier), and the active secretion of drugs and metabolites into the bile and/or urine [2].

Drug metabolizing enzymes and transporters are often involved in clinically significant drug–drug interactions. The mechanism of this interaction often involves drug-induced increases in enzyme or transporter activity (induction). As a consequence, disposition of other drugs that are metabolized or transported by the induced protein will change, possibly resulting in an adverse event. The induction of drug-metabolizing enzymes and transporters is mediated by a group of receptors known as orphan nuclear receptors. Chemical interactions with these receptors and the consequences of these interactions are reviewed here.

PXR and CAR: prototypic xenobiotic orphan nuclear receptors

Orphan nuclear receptors belong to the nuclear receptor (NR) superfamily of transcriptional factors. In most cases, these receptor proteins were identified without knowing their endogenous and/or exogenous ligands, so they were called 'orphan' receptors. Most, if not all, NRs share two essential functional domains that include the N-terminal DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) [3]. The conserved DBD consists of two DNA-binding zinc fingers and the LBD folds to form a hydrophobic pocket into which the ligand binds.

In 1998, the rodent orphan NR pregnane X receptor, PXR [4], and its human homolog hPXR (also known as steroid and xenobiotic receptor, SXR, or PAR [5–7]), were isolated as candidate xenobiotic receptors postulated to regulate *CYP3A* gene expression. It took this name because pregnenolone and its derivative, pregnenolone 16α-carbonitrile (PCN), can activate PXR. Another orphan receptor, constitutive androstane receptor (CAR), was cloned several years earlier [8] but its identity as a xenobiotic receptor was not appreciated until the discovery that its constitutive activity can be inhibited by selective androstane metabolites [9]. The role of CAR in positive xenobiotic regulation of *CYP2B* genes was first shown in 1998 [10].

Xenobiotic receptors, such as PXR and CAR, regulate gene expression by forming heterodimers with the retinoid X receptor (RXR). The regulation is achieved by binding of the PXR–RXR or CAR–RXR heterodimers to the specific xenobiotic response elements (XREs) present in the promoter regions of drug-metabolizing enzymes and transporters (Figure 1). PXR is activated by a variety of xenobiotics

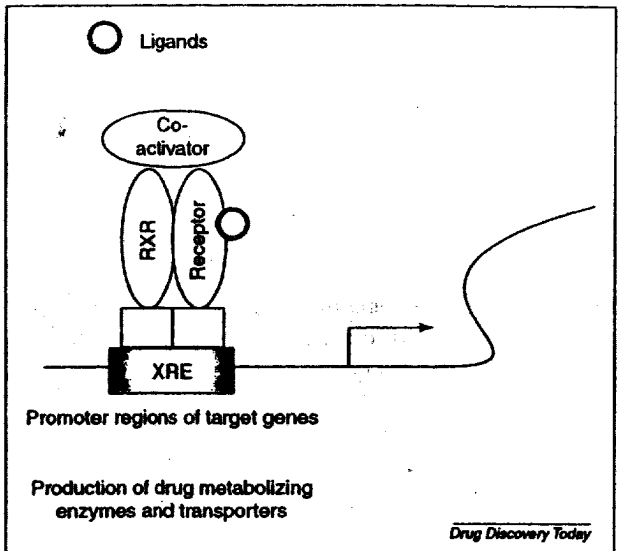

Figure 1. Xenobiotic receptor-mediated regulation of drug-metabolizing enzymes and transporters. Activation of xenobiotic receptors, such as pregnane X receptor (PXR) and constitutive androstane receptor (CAR), induces phase I and II enzymes and drug transporters. This transcriptional activation requires: (1) binding of ligands and recruitment of co-activators; (2) formation of heterodimers with retinoid X receptor (RXR); and (3) binding of the heterodimers to the xenobiotic response elements (XRE) in the target gene promoters.

including drugs known to induce hepatic and intestinal CYP3A activity [4–7]. Although CAR shows relatively high basal activity to transactivate genes without ligand ('constitutive'), its activity can be inhibited by antagonists, such as androstane metabolites [9], and potentiated by agonists, such as phenobarbital (PB) and 1,4-bis[2-(3,5 dichloropyridyloxy)] benzene (TCPOBOP) [10–12].

The respective regulation of CYP3A and CYP2B by PXR and CAR has been firmly established via the generation of mice deficient in PXR and CAR [13–15]. Disruption of the mouse *PXR* locus by homologous recombination abolishes the CYP3A induction in response to PCN and dexamethasone [13,14]. Similarly, CYP2B induction in response to PB and TCPOBOP was completely eliminated in the CAR-null mice [15].

Subsequent functional analysis has revealed a much broader role of PXR and CAR in xenobiotic regulation. It became evident that both receptors can function as master regulators in regulating additional phase I and phase II enzymes, as well as drug transporters. The mechanism of this broad regulation is the presence of PXR and CAR response elements in the promoter regions of many of these enzyme and transporter genes ([16]). These include the phase I enzymes CYP2C8/9/19 [17,18] and CYP3A7, phase II enzymes

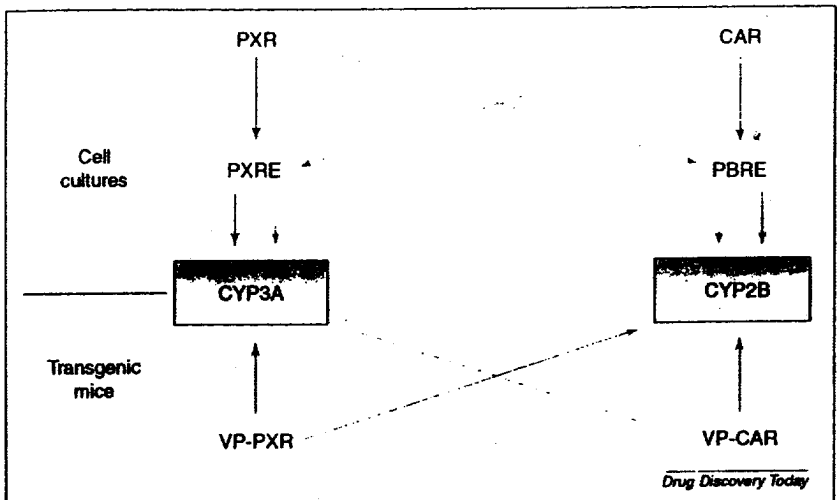

Figure 2. Cross-talk in xenobiotic nuclear receptor-mediated regulation of cytochrome P450 (*CYP*) genes. The reciprocal activation of xenobiotic response genes by pregnane X receptor (PXR) and constitutive androstane receptor (CAR) has been shown in cell cultures. In transgenic mice, activation of PXR induces *CYP3A* and *CYP2B*. In contrast, expression of activated CAR (VP-CAR) in mice induces *CYP2B* but not *CYP3A*. The green and red colored arrows indicate the direct and cross-regulation, respectively. Abbreviations: PXRE, PXR response element; PBRE, phenobarbital response element.

glutathione S-transferases (GSTs) [19], UDP-glucuronosyltransferases (UGTs) [20–22] and sulfotransferases (SULTs) [23,24], the transporters multidrug resistance protein 1 (MDR1) [17,25], MDR2 [26], multidrug resistance-associated protein 2 (MRP2) [27], and the organic anion transporter polypeptide 2 (OATP2) [14]. A broad role of PXR and CAR in xenobiotic regulation was further confirmed by several gene-profiling analyses performed in wild-type, transgenic and knockout mouse models [28,29].

Another unique functional feature of PXR and CAR is the overlap in the genes regulated by these receptors. For instance, PXR can regulate *CYP2B* genes and CAR can regulate *CYP3A* genes. The mechanism of cross-regulation has been shown to be due to shared response elements between receptors, as revealed by receptor-DNA binding analysis and transient transfection and reporter gene assays ([11,30–33], Figure 2). The generation of transgenic mice with hepatic expression of activated receptors enabled the evaluation of potential cross-regulation *in vivo*. The activated VP–PXR and VP–CAR were generated by fusing the VP16 activation domain of the herpes simplex virus to the N-terminal of the receptors. They shared similar DNA-binding specificities with their wild-type counterparts. Genetic activation of PXR *in vivo* caused sustained induction of CYP3A and CYP2B [11,13]. By contrast, in the VP–CAR transgenic mice, although CYP2B was induced as expected, the expression of *CYP3A* was largely unchanged or even slightly suppressed [24] (Figure 2). The lack of CYP3A11 induction in the VP-CAR mice was not due to the unresponsiveness of CYP3A11 in this transgenic line, as the expression of CYP3A11 in the VP–CAR mice remained inducible in response to the CAR ligand TCPOBOP [24].

Species specificity of xenobiotic regulation and the generation of 'humanized' mice

Xenobiotic induction of drug-metabolizing enzymes shows striking species specificity. For example, the antibiotic rifampicin (RIF) has been shown to be a CYP3A inducer in humans but not in rodents, whereas pregnenolone-16α-carbonitrile (PCN), an anti-glucocorticoid, is a rodent-specific CYP3A inducer. The species specificity of drug response has added another challenge in understanding the molecular basis of the regulation of drug-metabolizing enzymes and transporters. In the case of mammalian *CYP3A* gene regulation, previous pharmacological studies in primary cultures of hepatocytes suggest that it is not the promoter structure of the *CYP3A* genes that dictates the pattern of CYP3A inducibility but, rather, it must be a species-specific cellular factor(s). Accumulating evidence has established PXR and CAR as examples of these important cellular factors.

Although rodents are the standard laboratory models in the assessment of drug metabolism and toxicity, they probably are not reliable predictors of the human CYP enzyme inducibility due to the species-specificity of xenobiotic response. Using transfection and transgenic approaches, we have demonstrated that the species origin of the PXR receptor, rather than the promoter structure of *CYP3A* genes, dictates the species-specificity of CYP3A inducibility [13]. The species-specific ligand specificity has been thought to be due to the divergence of amino acid sequences in the ligand-binding domains of the human and mouse PXR receptors (Figure 3a). Four residues in the LBD of hPXR were shown to be crucial for interaction with the hPXR-specific ligand SR12813. When each of these residues was mutated to the corresponding hPXR amino acids, the mouse-human hybrid receptor showed a human-like ligand response profile [34]. The hypothesis that the species origin of the receptor is the determining factor for the species specificity of the ligand response led to the generation of 'humanized' mice, in which the mPXR was deleted via

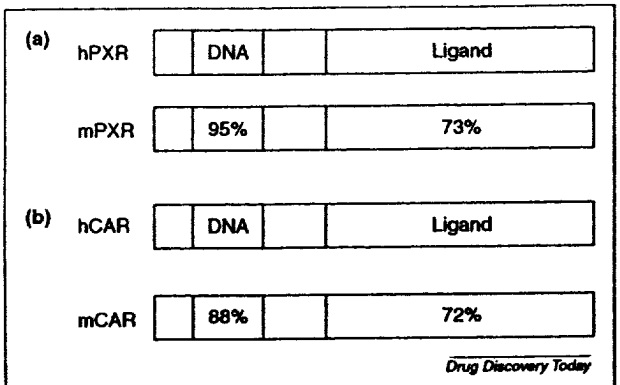

Figure 3. Sequence divergence between the human and rodent xenobiotic receptors. (a) Comparison of the human and mouse gene encoding PXR. (b) The human and mouse gene encoding CAR. The percentage nucleotide sequence identity in the DNA- and ligand-binding domains is indicated.

homologous recombination and hPXR was introduced into the mouse liver through a liver-specific transgene [13]. These mice exhibited a 'humanized' hepatic xenobiotic response profile, readily responding to the human-specific inducer RIF in a concentration range equivalent to the standard oral dosing regimen in humans [13]. The generation of these mice represents a major step toward generating a humanized rodent toxicological model that is continuously renewable and completely standardized. In addition, a PXR-mediated and mechanism-based transfection- and reporter-gene system has also been shown to be an effective *in vitro* approach to screen for drugs that might be precocious hPXR activators. Although the *in vitro* screen is fast, the availability of hPXR 'humanized' mice offers a unique screening tool to evaluate drug–drug interactions *in vivo*. These humanized mouse models represent important steps in the development of safer human drugs.

The original humanized mice express hPXR exclusively in the liver [13]. The drug-metabolizing enzymes and xenobiotic receptors are also highly expressed in the intestinal tracts, therefore it is conceivable that mouse models with the humanized receptors expressed in the liver and intestine would represent a more complete humanized mouse. This can be achieved using a promoter that can target the expression of hPXR transgene to the liver and intestine. An alternative strategy is to 'knock-in' hPXR in the mouse locus. This would not only direct expression of hPXR both in liver and intestine, but also normalize expression levels and tissue patterns to the endogenous gene.

CAR, like PXR, also exhibits species-dependent ligand specificity, which might also be explained by the divergence in the LBDs between species (Figure 3b). Neither androstenol nor TCPOBOP, the respective mouse CAR antagonist and agonist, affects human CAR activity. Although moderately potent ligands for hCAR have been reported [35], none exhibited potency comparable with androstenol and TCPOBOP toward mCAR. Nevertheless, humanized CAR mice, analogous to the previously reported humanized hPXR mice, have been created [36]. CAR-null mice were resistant to acetaminophen toxicity but with introduction of hCAR, the sensitivity to acetamenophen toxicity was recovered [36]. hCAR was also shown to mediate xenobiotic induction of bilirubin-clearance enzymes [22].

Beyond PXR and CAR: xenobiotic receptor newcomers

In addition to PXR and CAR, the expression of genes encoding drug-metabolizing enzymes and transporters is also subject to regulation by other nuclear receptors, such as the vitamin D receptor (VDR), farnesoid X receptor (FXR), and the retinoid X receptor (RXR). Recent evidence includes the following.

- Activation of VDR by bile acids or vitamin D3-induced *CYP3A* gene expression [37–39]. The VDR-mediated induction of CYP3A, a bile-acid detoxifying enzyme, could account for the preventive effects of vitamin D on colonic carcinogenesis promoted by high-fat diets or toxic bile acids [38].
- FXR was also shown to regulate the expression of the gene encoding dehydroepiandrosterone sulfotransferase (*SULT2A9*) [40]. Interestingly, PXR, CAR and FXR regulate *SULT2A9* gene expression by sharing the same IR-0 (inverted repeat without a spacing nucleotide) response element found in the promoter of the rodent *SULT2A* genes.
- A liver-specific deletion of the *RXRα* locus in mice causes decreased basal expression of several *CYP* genes, including *CYP3A* [41], which is consistent with the notion that RXR is the obligatory heterodimerization partner for several xenobiotic receptors.

More recently, hepatic nuclear factor 4α (HNF4α) has been shown to determine PXR- and CAR-mediated xenobiotic induction of *CYP3A4*. The *CYP3A4* promoter activity, even in the presence of PXR or CAR, has been known to be most pronounced in liver-derived cells especially the primary hepatocytes, but minimal or modest in non-hepatic cells, suggesting that a liver-specific factor is required for physiological transcriptional response. HNF4α, a liver-enriched orphan receptor, has been proposed to be one such hepatic factor [42]. A specific *cis*-element was identified in the 5′ regulatory sequences of the *CYP3A4* gene, which confers HNF4α binding and permits PXR- and CAR-mediated gene activation. Consistent with the role of HNF4α in *CYP3A* regulation, mice with conditional liver-specific

deletion of HNF4α, had reduced basal and inducible expression of *CYP3A* [42]. The key role of HNF4α in regulating PXR-mediated xenobiotic induction of liver enzymes in fetal livers was also reported independently by Kamiya *et al.* [43].

Beyond xenobiotics: PXR as an 'endobiotic receptor'

Although PXR has been identified as a 'xenobiotic receptor', emerging evidence has pointed to an equally important role of PXR as an 'endobiotic receptor' – that is, it responds to a wide array of endogenous chemicals. Moreover, the activation of PXR by endogenous ligands has implications in several important physiological and pathological conditions.

One family of endogenous PXR ligands identified shortly after the cloning of PXR are bile acids, the catabolic end products of cholesterol metabolism. Despite some beneficial function, excess accumulation of bile acids, such as the secondary bile acid lithocholic acid (LCA), has been shown to cause cholestasis (impaired bile flow) in experimental animals and has long been suspected of doing the same in humans. Xie *et al.* and Staudinger *et al.* showed that PXR acts as an LCA sensor and plays an essential role in detoxification of cholestatic bile acids [14,44]. Activation of PXR by bile acids or other xenobiotic inducers causes the induction of CYP3A, an enzyme that facilitates the detoxification of bile acids. Pretreatment of wild-type mice, but not the PXR-null mice, with PCN reduced the toxic effects of LCA. Moreover, genetic activation of PXR by expressing the activated PXR in the liver of transgenic mice was sufficient to confer resistance to the hepatotoxicity of LCA [44]. Consistent with the notion that activation of PXR facilitates bile-acid detoxification, increased serum levels of bile acids have been suggested to be a factor in the development of pruritis and studies in humans have shown that PXR activator RIF can be used to treat cholestasis-associated pruritis [45,46].

More recently, the bile acid intermediates formed during cholesterol catabolism have been shown to function as PXR agonists. The sterol 27-hydroxylase (CYP27A1) is an important enzyme in regulating the production of bile acids from cholesterol. In humans, mutations in the *CYP27A1* gene were responsible for the cerebrotendinous xanthomatosis (CTX), a genetic disease manifested by the accumulation of 25-hydroxylated bile alcohols, such as 25-tetrol, several 25-pentol isoforms, and possibly hexols and heptols. The clinical hallmarks of the disease include a marked deposit of sterols in a variety of tissues, a decrease in chenodeoxycholic acid production and associated mental retardation, premature atherosclerosis and tendon and brain xanthomas [47].

Surprisingly, the *CYP27*-null mice did not develop the clinical manifestations of CTX [48,49]. This might be due to a dramatic increase in the expression of *CYP3A* with a resultant increase in the CYP3A-mediated hydroxylation and clearance of bile acid intermediates [50–52]. The increase in CYP3A enzyme production in the *CYP27A*-null mice has been reasoned to be due to the activation of mouse PXR by these bile acid intermediates, among which are three potentially toxic sterols, 7α-hydroxy-4-cholesten-3-one, 5β-cholestan-3α,7α.12α-triol, and 4-cholestan-3-one. Interestingly, these intermediates are more potent inducers toward mPXR than hPXR, which might explain, at least in part, why humans lacking functional CYP27A1 do not display a compensatory increase in CYP3A activity [50,51]. These reports establish the existence of a feed-forward regulatory or salvage pathway, in which potentially toxic bile acid intermediates activate PXR and induce their own metabolism and clearance to avoid accumulation.

In addition to bile acids and their intermediates, Vitamin K_2 has recently been shown to be a hPXR/SXR agonist and able to induce the expression of PXR target genes such as *CYP3A4* [53]. Interestingly, Vitamin K_2 treatment of osteosarcoma cells increased mRNA levels for the osteoblast markers, including bone alkaline phosphatase, osteoprotegerin, osteopontin and matrix Gla protein, suggesting a potential novel role of PXR in bone homeostasis [53].

Implication of xenobiotic regulation in human diseases

The implication of PXR- and CAR-mediated gene regulation in drug metabolism and drug interactions has been recognized since the first cloning of these xenobiotic receptors. Consistent with the notion that these enzymes and transporters are also implicated in the biotransformation and homeostasis of many endogenous chemicals that can influence physiological and pathological processes, accumulating evidence has pointed to a role of orphan receptor-mediated xenobiotic regulation both in normal physiology and in disease states.

Bilirubin clearance and jaundice

Bilirubin is the catabolic byproduct of heme proteins, such as β-globin and CYP enzymes. Accumulation of bilirubin in the blood is potentially hepato- and neuro-toxic. For example, an insufficiency in expression of UGT1A1, a key enzyme for the conjugation of bilirubin in Crigler-Najjar syndrome and Gilbert's disease results in severe hyperbilirubinemia. Deficiency of MDR2, a transporter protein responsible for the hepatic excretion of conjugated bilirubin, leads to Dubin-Johnson syndrome, characterized by the accumulation of glucuronidated bilirubin. Both PXR

and CAR have been shown to induce the expression of *UGT1A1* [20–22] and this has been proposed to explain why the transgenic mice expressing a constitutively active form of hPXR had twice the bilirubin clearance of the wild-type mice [21]. Although it remains to be confirmed in transgenic mice, it is possible that PXR and CAR promote the clearance of bilirubin by increasing the expression of multiple key components in the clearance pathway. In addition to UGT1A1, PXR and CAR have been shown to induce the expression of the genes encoding OATP2, GSTA1 and 2 and MRP2. OATP2 facilitates bilirubin uptake from blood into hepatocytes [54]. GSTA1 and 2 reduce bilirubin back efflux from hepatocytes into blood. Interestingly, Huang *et al.* showed that CAR expression is low in human neonates. This functional deficit might be a factor in neonatal jaundice seen in almost 60% of infants and explain the effectiveness of PB for the treatment of this condition [22].

Detoxification of bile acids

Bile acids are the major products of cholesterol catabolism in the liver. Despite their beneficial role in solubilizing biliary lipids and promoting their absorption, accumulation of bile acids can cause irreversible liver damage, resulting in cholestasis [55]. PXR has been shown to be protective against bile acid hepatotoxicity. Both pharmacological (using PCN) and genetic activation of PXR in mice was sufficient to confer resistance to toxicity by LCA [14,44]. By contrast, mice deficient in PXR showed heightened LCA toxicity. The PXR-mediated protection was originally thought to be due to the induction of CYP3A [44]. Subsequent studies suggest that the induction of hydroxysteroid sulfotransferase (*SULT*), another PXR target gene, might also play a role in this protection [23,56]. More recently, Saini *et al.* [24] reported a novel CAR-mediated and CYP3A-independent pathway of bile acid detoxification. Using transgenic mice bearing conditional expression of the activated CAR, Saini *et al.* demonstrated that activation of CAR is both necessary and sufficient to confer resistance to the hepatotoxicity of LCA [24]. Surprisingly, the CAR-mediated protection is not due to the expected and previously characterized CYP3A pathway but, rather, is associated with a robust induction of *SULT* gene expression and increased LCA sulfation. Interestingly, activation of CAR was also associated with an increased expression of the 3′-phosphoadenosine 5′-phosphosulfate synthetase 2 (PAPSS2), an enzyme responsible for generating the sulfate donor PAPS [24]. However, it is not clear whether or not PAPSS2 is a direct transcriptional target of CAR. Analysis of gene knockout mice revealed that CAR is also indispensable for ligand-dependent activation of SULT and PAPSS2 *in vivo*.

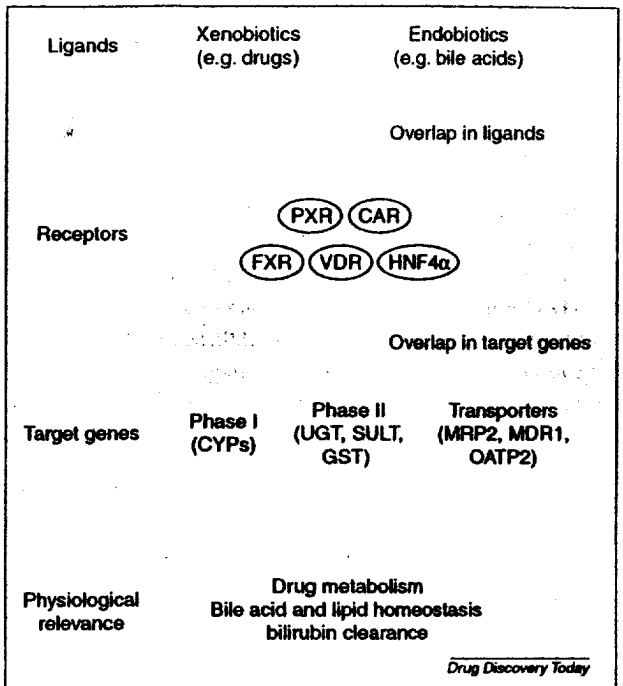

Figure 4. The complexity of mammalian xenobiotic response and its regulation by xenobiotic nuclear receptors. The activation of nuclear receptors by xenobiotic and endobiotic ligands, and subsequent regulation of phase I and phase II enzymes and drug transporters will eventually affect many physiological and pharmacological responses, such as drug metabolism and the homeostasis of bile acids, lipids and bilirubin. Abbreviations: PXR, pregnane X receptor; CAR, constitutive androstane receptor; FXR, farnesoid X receptor; VDR, vitamin D receptor; HNF4α, hepatic nuclear factor 4α; CYPs, cytochrome P450 family; UGT, UDP-glucuronosyltransferases; SULT, sulfotransferase; GST, glutathione S-transferases; MRP2, multidrug resistance-associated protein 2; MDR1, multidrug resistance protein 1; OATP2, organic anion transporter polypeptide 2.

Therefore, CAR has been established to play an essential and unique role in controlling the mammalian sulfation pathways and to facilitate bile-acid detoxification. It is important to note that several other orphan receptors, such as FXR and SHP, also play a crucial role in the homeostasis of bile acids [57–59], but this is beyond the scope of this review.

Summary and perspective

PXR and CAR are two orphan receptors originally identified as 'xenobiotic receptors' that regulate CYP gene expression. Subsequent studies have revealed much more complex regulatory pathways governed by these receptors, as summarized in Figure 4.

- Both receptors can function as master regulators to control the expression of phase I and phase II drug-metabolizing

enzymes, as well as members of the drug transporter families.

- Additional nuclear receptors, such as FXR, VDR and HNF4α, have also been shown to participate in the regulatory network.
- There is significant cross-talk among xenobiotic receptors, as manifested by overlap in xenobiotic ligands and target genes. This cross-talk is believed to be the molecular basis for the fail-safe xenobiotic regulatory networks that facilitate host protection.
- Additional functions of these receptors have been identified. A notable function is the establishment of these receptors as 'endobiotic receptors' that respond to a wide array of endogenous chemicals.
- Due to the pleiotropic function of drug-metabolizing enzymes and transporters, the implication of xenobiotic receptor-mediated regulatory pathways has been shown to be far beyond drug metabolism and drug-drug interactions. Additional physiological roles include bile-acid detoxification and bilirubin clearance.

It appears that PXR-controlled xenobiotic regulation is a double-edged sword. One of the remaining challenges is to find out whether the biological actions of PXR make this receptor suitable as a drug target for treatment of human diseases, such as bile acid-associated cholestasis, and for chemoprevention of colon cancers. Both RIF and the herbal remedy St John's Wort have been empirically used to treat cholestatic liver diseases [16]. The relief from cholestasis-associated pruritis and amelioration of cholestasis by RIF was associated with increased 6α-hydroxylation of bile acids, which in turn facilitates glucuronidation by the UGTs at the 6α-hydroxy position. RIF and St John's Wort are both potent agonists of hPXR and *CYP3A* and *UGT* are both PXR target genes, suggesting that the anti-cholestatic effects are mediated by PXR receptor.

Xenobiotic receptors mediate pharmacological and genetic control of the expression of drug-metabolizing enzymes and transporters, therefore the identification of PXR and CAR opens up a new perspective in pharmacogenetics and pharmacogenomics. Pharmacogenetics has traditionally focused on the polymorphism within the coding sequences of genes that encode various enzymes and transporters. Having enhanced our understanding of pharmacogenetics, the cDNA polymorphisms might not explain all of the inter-individual and inter-race variations in enzyme activity. The identification of xenobiotic nuclear receptors leads to several important questions from a pharmacogenomic perspective: (1) are there natural allelic variants of PXR or other xenobiotic receptors that exhibit differential transactivation potency to induce enzymes and transporters? (2) Are there polymorphisms in the promoter regions of target enzyme or transporter genes that might alter the binding affinity of xenobiotic receptors? Recent reports appear to support these notions [60–62]. However, we believe many more comprehensive studies are needed before this pharmacogenomic information can be applied to develop truly 'personalized' medicine.

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